RELATION OF LENGTH OF DAY TO GROWTH OF TIMOTHY [1]

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INTRODUCTION

Since 1920, when Garner and Allard [2] first announced their discovery of the principle of photoperiodism, many investigators have published results of studies of this phenomenon as it applies to many kinds of plants.[3]

In some plants, earliness and lateness are not the only characteristics that are affected by changes in the length of day. It has been shown that in timothy (*Phleum pratense* L.) not only time of blooming but also elongation of the internodes of the stems and the number of elongated internodes per culm are affected by the length of day.[4]

In a preliminary test conducted in 1930 it was found that timothy plants that bloom and mature early when grown under natural conditions respond to days artificially made of uniform lengths in a different way from plants   development. This discovery suggested the   **316785**   ig an experiment for the purpose of determinii ||||||||||||||||||||||||||||| othy plants that bloom and mature at differer   ige in length by uniform gradations from relati   **IARI**   long.

When plants propa₵   a single original timothy plant are grown at stations in different latitudes, it has been found that in the Northern Hemisphere the season for blooming of these plants does not progress from south to north at the uniform rate per day of one quarter of a degree, as predicted in Hopkins' bioclimatic law.[5] On the contrary, the plants bloom relatively later at the southern stations and relatively earlier at the northern stations than this law prescribes.[6] One possible explanation for this is that the development of the plants in the South during early spring is delayed by the relatively short days that occur in southern latitudes, and in northern latitudes the development is hastened by the relatively long days that occur during late spring and early summer.

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MATERIAL AND METHODS

For this investigation selected timothy plants were used. These plants are referred to as strains of timothy. All the strains listed in tables 2 and 3, except the last four, were selected from plants grown from seed of American origin; the last four originated from seed from northern Europe and are later than plants ordinarily occurring in meadows in the United States. These strains have been arranged in a series, according to the dates when the first florets bloomed on plants growing with natural illumination at Washington, D.C., in 1931. They represent uniform gradations from the earliest to the latest. The experiment was conducted at the Arlington Experiment Farm, Rosslyn, Va., near Washington, D.C.

On December 16, 1930, each of several plants was divided, and each subdivision, bearing the same number that had been assigned the original plant, was placed in a 3-inch pot. On January 26, 1931, each plant was transferred to a 4-inch pot. The plants were grown in a cool greenhouse (50° to 55° F.) until February 18, 1931, when they were removed to coldframes built upon trucks on tracks. These trucks were moved out of doors for 10 hours each day until the final tests under the various lengths of day began; during the remainder of the time they were kept in dark houses.

After the experiment was begun, plants of each strain were grown under all or part of the following numbers of hours of illumination each day: 10, 12, 12.5, 13, 13.5, 14, 14.5, 15, 16, 17, and 18. In addition, one or more plants of each strain, used as controls, were grown under the natural lengths of day.

The length from sunrise to sunset of the longest day occurring at Washington is 14 hours and 54 minutes. The plants grown with uniform lengths of day less than this maximum were placed in metal cans or buckets on trucks, which were moved into ventilated dark houses for that part of each day during which the plants received no illumination. The plants grown with 15 hours or more of illumination each day were placed out of doors, where the length of day was extended as desired by means of electric lights placed over the plants.

The plants that received 14.5 hours or less of illumination were out of doors each day for the periods indicated in table 1, from the date the test began until the final records were obtained.

TABLE 1.—*Time during which timothy plants receiving illumination for 10 to 14.5 hours daily were out of doors*

Date when test began	Length of period of illumination each day	Hours during which plants were out of doors	Date when test began	Length of period of illumination each day	Hours during which plants were out of doors
	Hours			*Hours*	
Apr. 11	10	From 6 a.m. to 4 p.m.	May 18	13.5	From 5 a.m. to 6:30 p.m.
Do	12	From 6 a.m. to 6 p.m.	Do	14	From 5 a.m. to 7 p.m.
Do	12.5	From 6 a.m. to 6:30 p.m.	Do	14.5	From 5 a.m. to 7:30 p.m.
Do	13	From 5 a.m. to 6 p.m.			

Artificial light was required in order to obtain constant illumination for 15, 16, 17, and 18 hours each day, periods representing approximately the maximum lengths of day for the latitudes 41°, 48°, 53°, and 57°, respectively. This added illumination was furnished for

each group of plants by four 200-watt gas-filled tungsten lights with reflectors, built according to standard specifications. These lights were placed at each corner of a square of such dimensions that the distance from center to center of the lights was 3 feet. The lights, which were kept about 2 feet above each group of plants, were arranged on an adjustable frame so that they could readily be raised as the plants increased in height. Each set of plants was screened by partitions high enough to cut off the direct rays of its group of lights from all the other sets of plants.

In order to maintain illumination for a uniform period each day, it was necessary to use electric lights for different lengths of time. Constantly decreasing daily periods of artificial light were required up to the midsummer solstice, and constantly increasing periods of light after the solstice. Four electric time switches were used to control the required decrements or increments of artificial light, and the necessary changes were made each day. The tests under all four lengths of day, from 15 to 18 hours, began April 11. The lights were turned on at 5 p.m., and were kept on for different periods, so that the different groups of plants were illuminated for 15, 16, 17, and 18 hours, respectively, from sunrise each day.

EXPERIMENTAL RESULTS

EFFECTS OF DIFFERENT LENGTHS OF DAY ON DIFFERENT PHASES OF GROWTH

Insofar as vegetative growth is concerned, the plants thrived under all lengths of day within the range of this experiment. There were, however, great variations (1) in the time when the heads emerged from the enclosing leaf sheaths, (2) in the time of occurrence of the flowering process, (3) in the characteristics of the stems, and (4) in the development of the stems.

TIME WHEN HEADS EMERGE FROM ENCLOSING LEAF SHEATHS

The date of emergence of the earliest head of each plant from within the enclosing leaf sheath is shown in table 2.

In general, the later in the season the heads appeared on plants grown with natural illumination, the greater was the number of hours of illumination required for the development of the inflorescences on plants of the same strain grown with artificial light. Plants of the 2 strains that were earliest under natural conditions produced inflorescences when subjected to only 10 hours of light each day. Plants of 1 of the 4 strains that were latest under natural illumination required day lengths of 14.5 hours, while none of the plants of the other 3 strains of this group produced inflorescences under less than 15 hours of illumination daily. Plants of the other strains had a minimum light requirement of between 10 and 14.5 hours each day for the development of inflorescences; in general, the minimum number of hours of illumination required tended to increase gradually as the time of heading became later on the plants of these strains when grown under natural conditions.

As the number of hours of illumination each day increased, the date of heading on the plants of any strain gradually became earlier until the optimum day length had been attained; after this, even with continued increase in the length of day, the date of heading remained practically the same. This statement may be illustrated by the

TABLE 2.—*Dates in 1931 when the first heads appeared on plants of different strains of timothy, grown with natural daylight from 10 hours to full length of day each day and with added artificial light to obtain illumination of 15 to 18 hours daily, near Washington, D.C.*[a]

Strain no.	Full day	Date of emergence[b] of first heads in plants grown with indicated hours of illumination										
		10	12	12.5	13	13.5	14	14.5	15	16	17	18
19456	May 20	June 22	May 18	May 18	May 20	(c) May 22	(c)	(c)	(c)	(c)	(c)	(c)
19457	May 25	June 23	June 5	June 3	June 3	(c)	(c)	(c)	(c)	(c)	(c)	(c)
19458	May 26			June 15	June 16	(c)	(c)	(c)	(c)	(c)	(c)	(c)
15092	May 28 / May 26		June 27	June 8	June 15	May 28 / do	May 28 / May 26	May 28 / May 25	May 18	May 15	May 13	May 13
11902	June 1		June 24	June 10	do	June 3	June 1	June 1	May 20	May 18	May 14	May 18
6127	June 8 / June 10 / June 12	July 20			June 25	June 18 / June 27	June 15	June 8	May 25	May 20	May 19	do
6743	June 3 / June 6	July 20		June 26	June 27	June 11	June 8	June 4	do	June 1	do	May 15
11966	June 13 / June 16				July 22	July 3 / June 23	June 22 / June 15 / June 26 / June 30	June 17 / June 13	May 29	May 21	do	May 16
9220	June 18		Aug. 3 / July 31	July 21	Aug. 3	July 7 / July 13 / July 14	do	June 19	June 1	May 19	May 18	May 18
12421	June 19			July 11	July 13	July 22	July 9	June 22	June 5	May 25	May 20	May 21
15485	June 25 / June 26 / June 27							June 30	June 8	May 26	May 22	May 22
19416	June 30						July 10	June 27	do	do	May 21	May 21
15445	July 3 / July 7 / July 3 / July 6								June 20	June 1	May 26	May 26
19459	July 11							July 20	June 13	May 29	May 23	May 21
19460	do								June 16	June 1	do	May 23
19461	July 15 / July 13 / July 17								June 18	June 3	May 25	May 25

[a] To obtain daily exposures of 10 to 14.5 hours required keeping the plants in a darkened house for a certain number of hours each day; to obtain daily exposures of 15 to 18 hours required the use of artificial light to supplement the normal length of day.

[b] Leaders indicate that no heads emerged under the period of illumination specified; more than 1 date of emergence or line of leaders merely indicates that the test included 2 or more sets of plants.

[c] No plants were grown under this period of illumination.

records of the plants of strain no. 9220 (table 2). Under natural conditions, the first head appeared on the control plant on June 18. On the plant grown with 10 hours of light each day, no inflorescence developed; on that grown with 12 hours of light the tip of the first head appeared on July 31. With increasing length of day the time of heading gradually became earlier, until with 16 hours of illumination the time of heading occurred on May 19. When exposed to 17 and 18 hours of light each day, the heads of the plants appeared on May 18, practically the same date as on the plant grown under a 16-hour day.

<div align="center">TIME OF FLOWERING</div>

The relative dates at which the first florets appeared on plants of the different strains grown with different periods of illumination corresponded, in a general way, to the relative dates at which the first heads had appeared on the same plants about 12 to 15 days earlier.
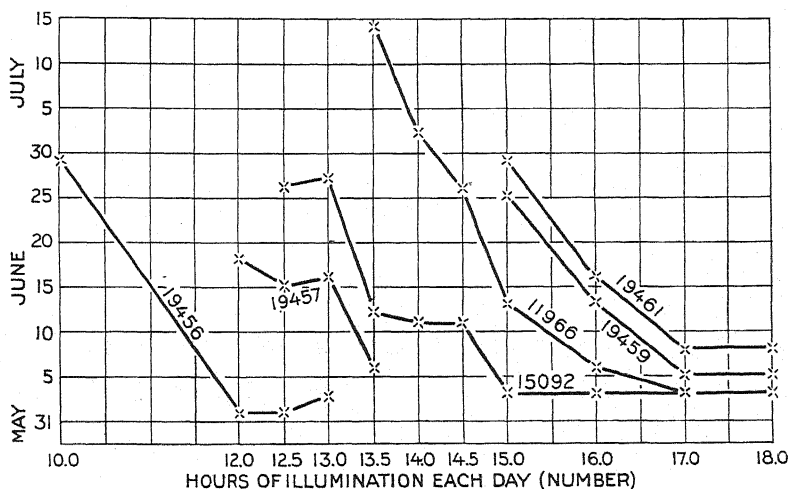

FIGURE 1.—Dates in 1931 when the first florets bloomed on different timothy strains (indicated by numbers), grown under various constant daily periods of illumination.

In plants that under natural conditions range gradually from very early to very late the response to days of different lengths, as indicated by the time of flowering, is quite consistent (table 3). On the control plant of strain no. 19456, the first florets bloomed on June 3; this was the earliest plant to bloom under natural conditions. On the plant of this strain grown with 10 hours of light each day, the first florets bloomed on June 29. As the date of flowering became later on plants of the different strains grown under natural conditions, the minimum number of hours of illumination under which any florets bloomed gradually increased, until, in the case of strain no. 19461, which was the latest to bloom under natural illumination, no florets appeared on plants grown under days of uniform length of less than 15 hours.

As the length of day increased, the dates on which the florets on the plants of any particular strain bloomed gradually became earlier up to the optimum period of illumination, after which there was no further increase in earliness. This tendency is shown by the records in table 3 and the curves in figure 1. Under natural conditions, the first florets

TABLE 3.—*Dates in 1931 when the first florets opened on plants of different strains of timothy, grown with natural daylight from 10 hours to full daylight each day and with added artificial light to obtain illumination of 15 to 18 hours daily, near Washington, D.C.*[a]

Strain no.	Date of first flowering [b] of plants grown with indicated hours of illumination											
	Full day	10	12	12.5	13	13.5	14	14.5	15	16	17	18
19456	June 3	June 29	June 1	June 1	June 3	(c)	(c)	(c)	(c)	(c)	(c)	(c)
19457	June 8		June 18	June 15	June 16	June 6	(c)	(c)	(c)	(c)	(c)	(c)
19458	June 11			June 27	June 26	(c)	(c)	(c)	(c)	(c)	(c)	(c)
15092	{ June 12 / June 13		July 14	June 22	June 27	June 12	{ June 11 / June 13	{ June 11 / June 12	June 3	June 3	June 3	June 3
11902	{ June 13 / June 17		Aug. 4	July 13	June 29	do	June 16	June 18	June 6	do	do	Do.
6127	{ June 18 / June 19				July 10	June 19	June 26	June 22	June 9	June 5	do	Do.
6743	{ June 23 / June 23				July 17	{ June 29 / July 6	June 29	June 19	June 12	June 6	do	Do.
11966	{ June 22 / June 26					June 25	{ June 29 / July 2	{ June 26 / June 29	June 13	June 16	do	Do.
9220	{ June 30 / June 29				Aug. 1	{ July 14 / July 20	{ do / July 13	July 2	June 15	June 5	do	June 4
12421	{ July 2 / June 30			July 18		July 23	do	July 3	June 15	June 8	June 5	June 4
15485	{ July 6 / July 7						July 20	July 9	June 20	do	do	June 5
19416	{ July 9 / July 11							July 11	June 22	do	do	June 4
15445	{ July 14 / July 20								July 2	June 16	June 12	June 8
19459	{ July 17								June 25	June 13	June 5	June 5
19460	{ July 20 / July 22 / July 25							July 29	June 29	June 16	June 8	June 4
19461	{ do / July 27								do	do	do	June 8

[a] To obtain daily exposures of 10 to 14.5 hours required keeping the plants in a darkened house for a certain number of hours each day; to obtain daily exposures of 15 to 18 hours required the use of artificial light to supplement the normal length of day.

[b] Leaders indicate that no florets opened under the period of illumination specified; more than 1 blooming date or line of leaders merely indicates that the test included 2 or more sets of plants.

[c] No plants were grown under this period of illumination.

on plants of no. 11966 bloomed on June 26. When the plants were grown under days 10 to 13 hours long, no florets bloomed; when they were grown with 13.5 hours of light, florets began to bloom on July 14. As the length of day increased, the date of blooming became gradually earlier up to days of 17 hours, under which the earliest florets bloomed on June 3. When the length of day was increased from 17 to 18 hours, the date of earliest bloom remained the same, June 3. There was a similar response to days of different lengths in the plants of other strains.

The later the plants of any strain of timothy grown under natural conditions produce inflorescences and florets in bloom, the greater is the length of day required for normal development when the plants are grown with days of various constant lengths. This statement is illustrated in figures 2 to 6, which show the plants as they appeared on July 1. The plants of strain no. 19456, the earliest under natural conditions, when grown with 12 hours of light each day produced
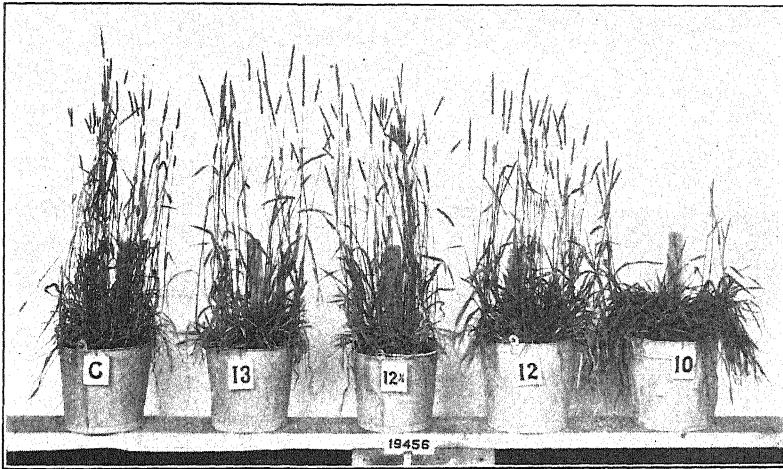

FIGURE 2.—Plants of timothy strain no. 19456, grown under different lengths of day, as indicated by number of hours on containers. The control plant (*C*) was grown under natural length of day. Photographed July 1, 1931.

inflorescences on which florets had bloomed before July 1. The plants of strain no. 15092 required a day of about 13.5 hours, and those of strain no. 6127 a day of 14 hours for normal growth. The plants of no. 19461, which was the latest strain used in this experiment, required a day of 16 hours for the production of normal culms with inflorescences.

If the natural length of day is too short for the development of culms and inflorescences of any timothy plant, it may be artificially increased, by means of electric light, to the daily number of hours of illumination which the plant requires. This is illustrated in figure 7, which shows plants of strain no. 19461 grown with natural illumination and with 15, 16, 17, and 18 hours of light each day. On the plants grown with natural illumination no inflorescences appeared until July 13; whereas on the plants grown with 16, 17, and 18 hours of light each day, normal culms and inflorescences had developed before June 25.

FIGURE 3.—Plants of timothy strain no. 15092, grown under different lengths of day, as indicated by number of hours on containers.   The control plant (C) was grown under natural length of day.   Photographed July 1, 1931.

FIGURE 4.—Plants of timothy strain no. 6127, grown under different lengths of day, as indicated by number of hours on containers. The control plant (C) was grown under natural length of day. Photographed July 1, 1931.
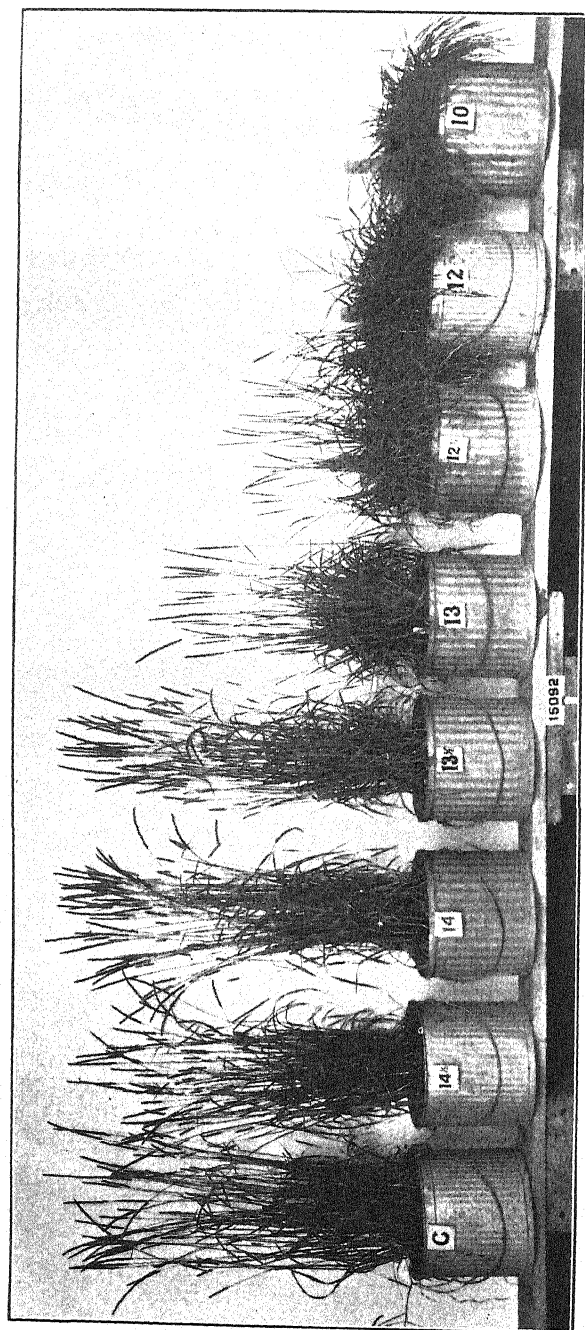
FIGURE 5.—Plants of timothy strain no. 15485, grown under different lengths of day, as indicated by number of hours on containers. The control plant (C) was grown under natural length of day. Photographed July 1, 1931.
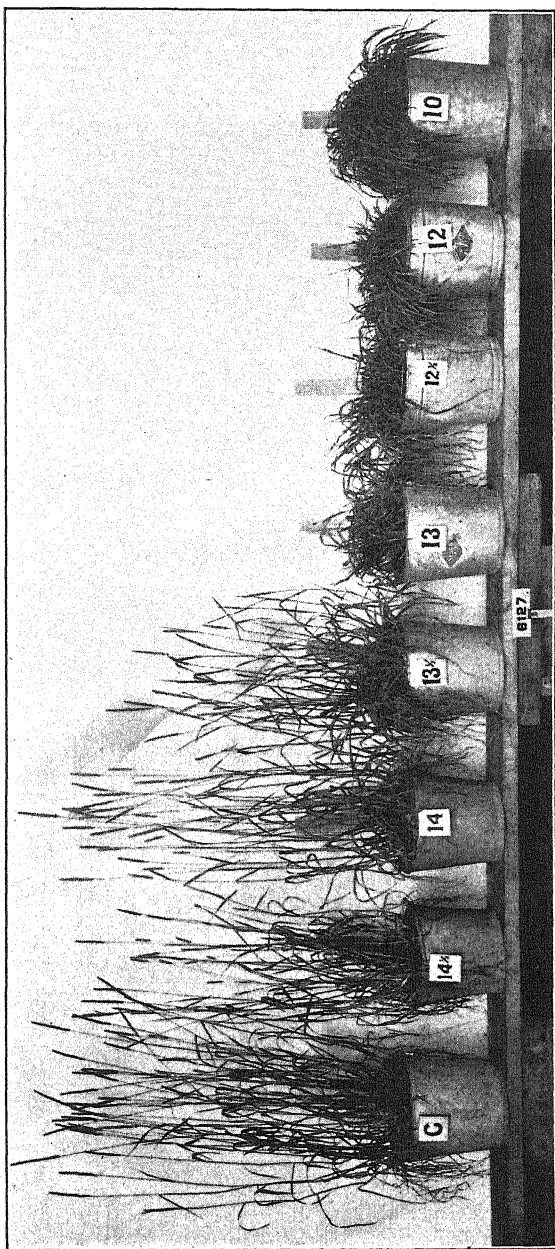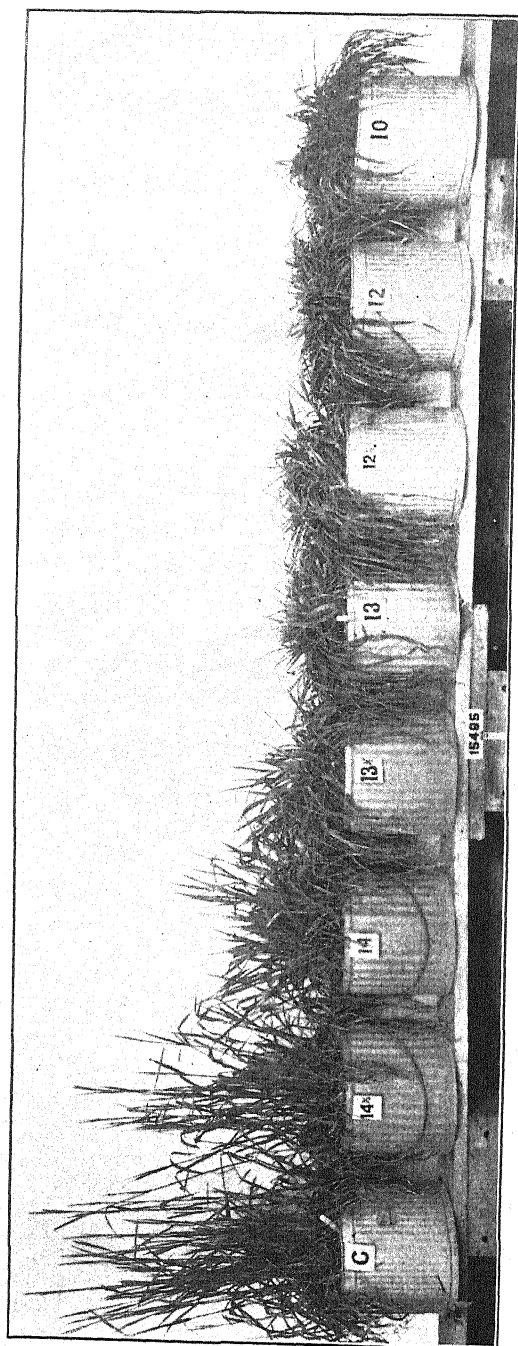
CHARACTERISTICS OF STEMS

The stems of plants which are grown with sufficiently long periods of illumination each day to enable the inflorescences to develop in a normal manner and on which the florets bloom at a normal time usually grow upright.   In this experiment the plants that were grown under days too short for normal development of the inflorescences were commonly characterized by stems which were declined or which were more or less procumbent at the base, sometimes bearing inflorescences with proliferations.   Figures 3 and 4 show that the plant of strain no. 15092 grown with 12.5 hours of illumination and the plant of strain no. 6127 grown with 13.5 hours of illumination daily had a tendency to a spreading habit of growth owing to the declined position of the stems.

On plants of all strains except those that were earliest under natural conditions, no elongation of the stems occurred under the shortest periods of illumination.   Thus, on the plants of no. 6127 (fig. 4), there was no elongation of stems under 10 to 13 hours of illumination daily; under 13.5 hours of illumination, elongation occurred although the stems were somewhat declined; on the plants grown under 14 and 14.5 hours of light each day and those grown under natural illumination, the stems grew in an upright position.

LENGTH OF STEMS

At the time of the first blooming of the florets of a timothy plant, the stems on which the inflorescences are borne have attained only a part of their final length.[7]

The data obtained in this experiment show that when the first florets bloomed the actual length of the stems varied according to the number of hours of daily illumination under which the plants had been grown.   The records show
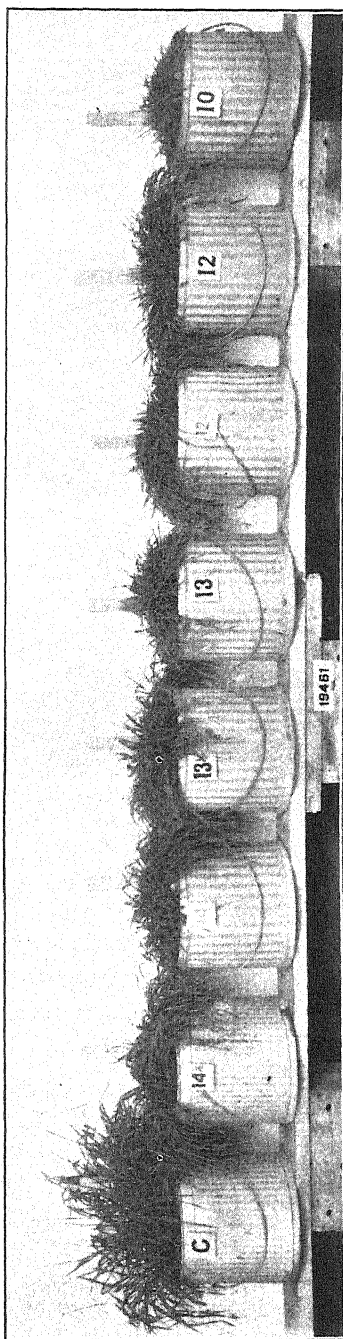

FIGURE 6.—Plants of timothy strain no. 19461, grown under different lengths of day, as indicated by number of hours on containers. The control plant (C) was grown under natural length of day. Photographed July 1, 1931.

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[7] EVANS, M. W. Pp. 10–13.   (See footnote 4.)

that generally, as the length of day increased, the length of the stems of the plants of any strain on the date when the first florets bloomed gradually increased to the maximum. After this maximum had been reached, the length of the stems was not essentially changed by continued increase in the length of day. For example, when the plants of timothy no. 11902 were grown with 12 hours of light, the longest stem was 16 inches at the time the florets began to bloom; as the length of day increased, the length of stem at the time the first florets bloomed also increased by fairly uniform steps to a maximum length of 41 inches on the plant grown with 15 hours of light each day. On the plants grown with 16, 17, and 18 hours of illumination daily, the length of the longest stem at the time the first florets bloomed was either 40 or 41 inches, practically the same length as on the plant illuminated for 15 hours each day. Table 4 and figure 8
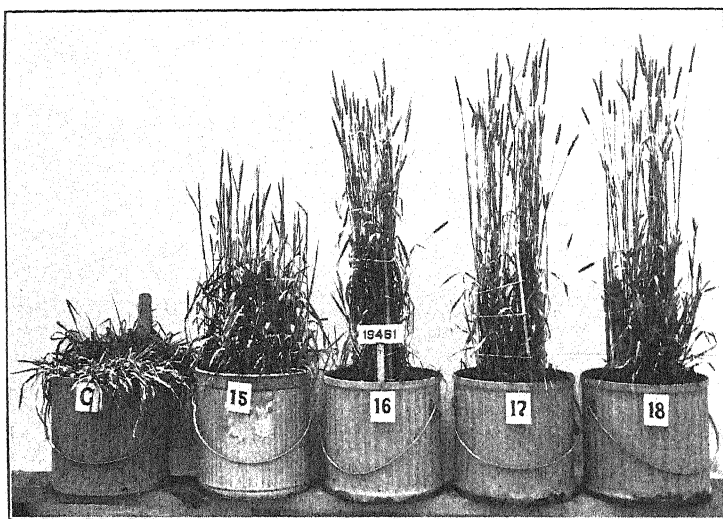

FIGURE 7.—Plants of timothy strain no. 19461 grown under natural length of day (C) and with days longer than normal, as indicated by number of hours on containers. This strain of timothy is the latest one (when grown under natural conditions) that was used in this experiment. Photographed June 25, 1931.

show the average length of the longest stems of the plants in groups of early, medium, and late strains of timothy grown under different lengths of day.

RESULTS OF CONTINUED GROWTH UNDER DAYS TOO SHORT FOR DEVELOPMENT OF CULMS AND INFLORESCENCES

In relation to those plants which were grown under days of short lengths and on which only vegetative growth had taken place prior to July 1, the question arises as to whether continued growth for a longer time under the same conditions would have resulted in the development of culms and inflorescences. The results obtained with the plants of strain no. 15485 are fairly typical and may be used to illustrate the general behavior, in this respect, of the plants of all the strains of timothy studied. Figure 9 shows the condition of the plants

of this strain on August 10, 6 weeks after the plants in figure 5 were photographed. On plants upon which partial development of culms occurred before July 1, there was a somewhat more advanced development on August 10. On plants that were grown with 10, 12, 12.5, and 13 hours of light each day, and on which only vegetative growth had taken place prior to July 1, no further development occurred as a result of 6 weeks of added growth. If timothy plants are grown, therefore, under days too short for the formation of culms and inflorescences these will not be formed even though the period of growth under the same light conditions is extended.
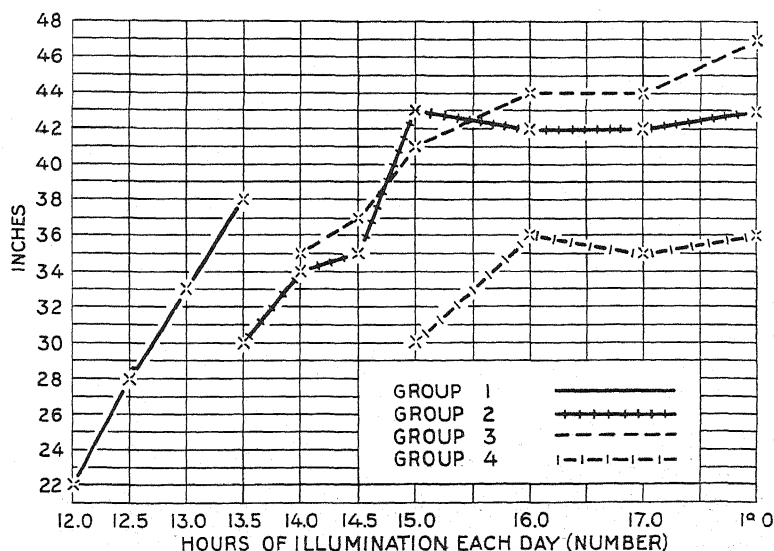

FIGURE 8.—Average length of the longest stem (in inches) of early (group 1), medium (groups 2 and 3) and late (group 4) timothy plants grown under various constant daily periods of illumination.

TABLE 4.—*Average length (inches), on the date when the first florets bloomed, of the longest stem of timothy plants grown under various constant daily periods of illumination and grouped according to relative earliness* [a]

Group	Average length (inches) of the longest stem grown under indicated hours of illumination daily [b]										
	Natu-ral	10	12	12.5	13	13.5	14	14.5	15	16	17	18
1	38	------	22	28	33	38	([c])	([c])	([c])	([c])	([c])	([c])
2	34	------	------	------	------	30	34	35	43	42	42	43
3	39	------	------	------	------	------	35	37	41	44	44	47
4	27	------	------	------	------	------	------	------	30	36	35	36

[a] When grown under normal conditions, plants of group 1 (nos. 19457 and 15092) were the earliest strains of timothy used in this experiment; those of group 2 (nos. 11902, 6127, and 11966) were the next earliest, those of group 3 (nos. 9220, 12421, and 15485) were next, and those of group 4 (nos. 15445, 19459, 19460, and 19461) were the latest.

[b] Leaders indicate that no average was available, because flowering either did not take place or some of the strains failed to flower under the indicated period of illumination.

[c] No average given because there were no plants of no. 19457.
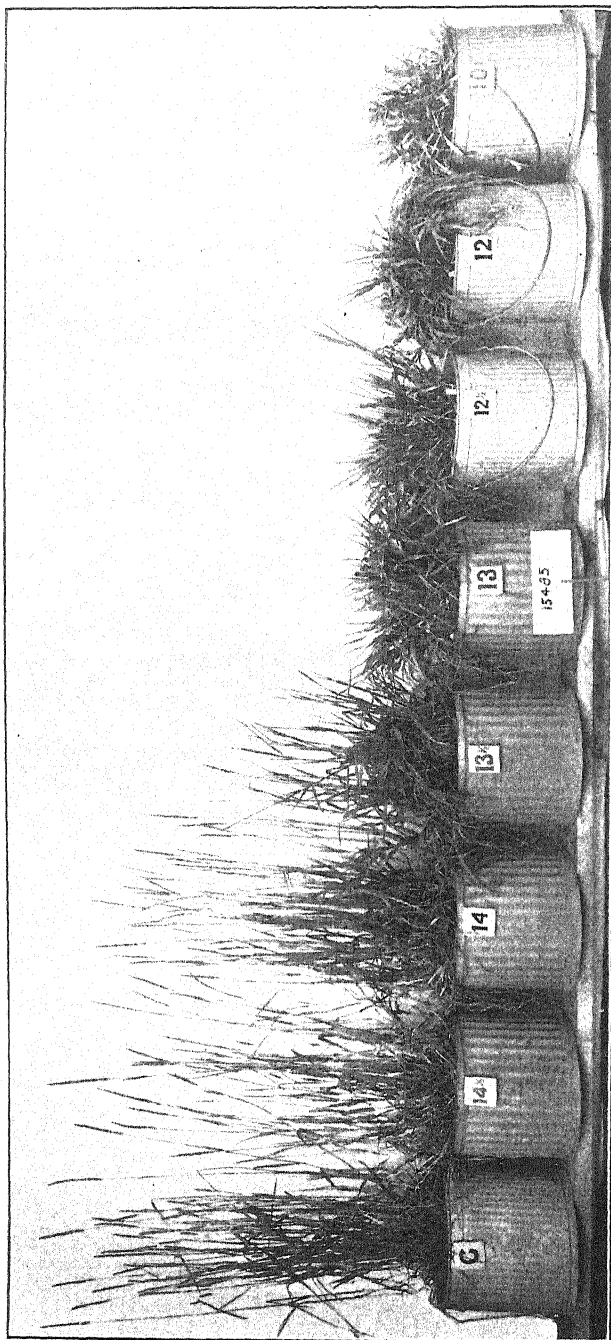
FIGURE 9.—Plants of timothy strain no. 15488, grown under different lengths of day, as indicated by number of hours on containers. The control plant (C) was grown under natural length of day. Photographed August 10, 1931. Some increase in the length of the stems on plants illuminated for 13.5 hours or more each day since July 1 may be observed by comparing this photograph with figure 5. Under 13 hours or less of light each day, no growth in length of the stems had taken place.

COMPARISON OF EFFECTS OF NATURAL AND ARTIFICIAL ILLUMINATION

The length of the longest day at Rosslyn, Va., where this experiment was conducted, is 14 hours and 54 minutes, or 14.9 hours. Tables 2 and 3 show that in most strains of timothy the heads appeared and the florets bloomed at approximately the same time on plants illuminated for 14.5 hours each day as on the control plants; on the plants grown under days 15 hours long, the heads appeared and the florets bloomed earlier than on the plants grown with natural illumination.

EARLINESS AND LATENESS IN TIMOTHY AN ADJUSTMENT TO DIFFERENT LENGTHS OF DAY OCCURRING AT DIFFERENT TIMES

In timothy, as in some other plants,[8] the earliness or lateness of different strains is evidently a matter of the adjustment of the plants to length of day. In figure 10 the strains of timothy are arranged according to the dates when the first florets bloomed on the plants grown with natural illumination; the first heads had appeared on these plants in practically the same order. Figure 10 also shows the dates on which the first heads appeared and the first florets bloomed on plants of most of these strains when grown with 18 hours of light each day, the maximum length of day employed. When the length of day was increased to 18 hours, plants of those strains that are late under natural conditions produced heads and florets in bloom al-

FIGURE 10.—Dates when the first head appeared and the first florets bloomed on timothy plants grown near Washington, D.C., under natural illumination and under 18 hours of illumination each day. Note that plants of those strains that are late under natural conditions in the latitude of Washington, D.C., when grown under 18 hours of daily illumination produced heads and florets in bloom nearly as early as plants of those strains that are early under natural conditions in the same locality.

most as early as plants of those strains that are early under natural conditions (fig. 10).

The results of this experiment indicate that in any locality some timothy plants produce inflorescences and florets in bloom earlier than other plants because they are adapted to relatively short days. On those plants that are adapted to longer days, the appearance of the heads and the flowering process are delayed until the days lengthen.

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[8] GARNER, W. W., and ALLARD, H. A. PHOTOPERIODIC RESPONSE OF SOYBEANS IN RELATION TO TEMPERATURE AND OTHER ENVIRONMENTAL FACTORS. Jour. Agr. Research 41: 719–735, illus. 1930.

SUMMARY

At the Arlington Experiment Farm, Rosslyn, Va. (near Washington, D.C.) there were grown under days of different lengths strains of timothy plants that when grown under natural conditions constitute a series ranging by fairly uniform gradations from very early to very late in the time their inflorescences appear, florets bloom, and seeds mature. In addition to the control plants grown under natural illumination, plants were grown with the periods of illumination artificially regulated by means of dark houses and electric lights to days 10, 12, 12.5, 13, 13.5, 14, 14.5, 15, 16, 17, and 18 hours long.

Insofar as vegetative growth is concerned, the plants grew well under all lengths of day within the range used in this experiment. There were great variations, however, with respect to the time of emergence of the heads from the enclosing leaf sheaths, the flowering process, and the characteristics and development of the stems.

The later the plants of different strains of timothy produce inflorescences and florets in bloom when grown under natural conditions, the greater is the length of day required for normal development when the plants are grown under days of different uniform lengths.

As the length of day is gradually increased above the minimum under which development of culms with inflorescences occurs, the stems lengthen and the time of the appearance of the inflorescences and the blooming of the florets gradually is shortened up to an optimum length of day. If the length of day is increased above this optimum, there is little added effect—at least up to 18 hours, the greatest length of day under which plants were grown in this experiment.

If the length of day is too short for the development of culms and inflorescences within the time that these processes ordinarily occur, continued growth of the plants for a longer time under the same length of day will not induce their development.

With a day of 14.5 hours, the timothy plants developed at about the same time as plants grown under the natural lengths of days occurring at Washington, D.C., which gradually increase up to a maximum of 14.9 hours at the summer solstice and then gradually decrease. When plants were grown under a day 15 hours long, they developed earlier than the control plants.

In timothy the earliness or lateness of different strains is evidently chiefly a matter of the adjustment of the plants to the lengths of day. The results of this investigation indicate that, in any locality, some timothy plants produce inflorescences and florets earlier than others because they are adapted to relatively short days. On those plants that are adapted to longer days, the appearance of the heads and the flowering process are delayed until, with the advancement of the season, the days increase to the lengths which these plants require for development.

DISTRIBUTION OF OXYGEN AND CARBON DIOXIDE IN MUSHROOM COMPOST HEAPS AS AFFECTING MICRO-BIAL THERMOGENESIS, ACIDITY, AND MOISTURE THEREIN [1]

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INTRODUCTION

At the present time stable manure is the medium almost universally used for cultivating the common mushroom (*Agaricus campestris* L.). Growers have learned from experience that a period of composting is necessary before the manure is suitable for making up into beds. A rapid fermentation during which comparatively high temperatures are generated is apparently desirable. In general the rate of decomposition of the manure and the suitability of the finished compost for mushroom growing seem to be largely dependent on the size and shape of the compost heap, its height and compactness, the quantity of water added during turning, the thoroughness of the mixing, and the number of days between turnings. These factors probably influence the condition of the finished compost primarily by establishing in the compost heaps conditions of aeration, moisture, and temperature, which in turn establish the trend of the development of the microbial and insect population of the heaps.

The studies described in this paper were undertaken in order to improve composting practice by learning something of the distribution and interaction of these physical and biological factors in typical mushroom compost heaps. At first the writers were concerned principally with recording the temperature, aeration, moisture content, and acidity in all parts of standard mushroom compost heaps. As the observations progressed it became apparent that conditions are radically different in different parts of compost heaps and that the factors of temperature, moisture, and acidity are dependent on aeration, presumably through its effect on microbial activity, in a roughly predictable manner. From these observations an attempt has been made to derive principles that will give the experimenter an approximate conception of the conditions of aeration, temperature, moisture, and acidity to be expected in all parts of stable manure composted as for mushroom culture in heaps of any size or shape.

METHODS

Ordinary soil thermometers were used for taking temperatures in compost heaps. After the metal tips of these thermometers are heated the instruments may be removed from the compost and read before the mercury begins to fall. For readings at depths greater than 1 foot it was necessary to add extensions to the handles. Complete immersion of these thermometers seems to cause little difference in the readings, and this may be determined and a correction applied. The use of ordinary thermometers of any type is not desirable for three reasons: (1) The fall of the mercury is very rapid once the instruments have been removed from the manure and it is consequently very difficult to take accurate readings; (2) in drawing the thermometers from the bottom of the heaps through the warmer interior the readings change materially; and (3) it is necessary to punch holes in the heaps larger than the diameter of the thermometers in order to drop them in, thus admitting currents of air that frequently change the temperature.

In taking temperatures the thermometers were placed at horizontal and vertical intervals of 6 inches or 1 foot in the heap, depending on the time available and the accuracy desired. After each reading the thermometers were cooled to about 80° F., put into the same holes, and shoved down to the next level. The readings obtained were recorded on crosshatched paper, and the temperature contours were filled in later.

Samples of air from within the compost heaps were obtained by means of a metal tube having a sharply pointed end. Just behind the point four holes were bored for the air to enter. This tube was connected with rubber hose to an Orsat gas-analysis apparatus and could be thrust into the pile at any point for sampling. The air was removed from the tubing before each sample was taken. Samples from within the heap were passed first through a solution of potassium hydroxide to absorb the carbon dioxide and then through alkaline pyrogallol to absorb the oxygen. The probable error of the Orsat apparatus under these conditions seemed to be about 0.2 percent. At times the error of sampling was probably several times this figure. A possible source of error is recognized in the presence within the compost heaps of gases, other than carbon dioxide and oxygen, that might be soluble in potassium hydroxide or alkaline pyrogallol. But in all probability the presence of such gases in small quantities would have little bearing on the problem under consideration and would not affect the evidence or the conclusions in any way.

The hydrogen-ion concentration of samples from different parts of the compost heap was determined with a portable potentiometer by the quinhydrone method. The determinations were made in the field so that the samples were tested only a few minutes after they were removed from the compost heap. There is a tendency for the readings of manure samples to drift toward the alkaline side after the quinhydrone is added. To equalize this effect all samples were allowed an equal period (4 minutes) between the adding of the quinhydrone and the taking of the readings. Preliminary tests showed an average difference of only 0.05 between the pH values of aliquot samples of compost tested with the hydrogen electrode and those tested with the quinhydrone electrode.

TESTS FOR CARBON DIOXIDE AND OXYGEN

Samples of air were taken at first from typical mushroom compost heaps at the Arlington Experiment Farm, Rosslyn, Va., 3 or 4 feet high and without artificial ventilation. Later, aspirations were made from commercial storage heaps 5 to 6 feet high, and from heaps with artificial ventilation at the ground level. In all heaps without artificial ventilation the oxygen content of the air in the interstices of the compost decreased steadily as the bottom center of the heap was approached from the sides or from the top. Anaerobic conditions were usually found within 2 or 3 feet from the side of the heap and 1 foot from the upper surface. Carbon dioxide analyses of the same samples of air indicated a corresponding increase in carbon dioxide as the lower center of the heap was approached. These phenomena are presumably due to the presence of an actively respiring microbial flora.
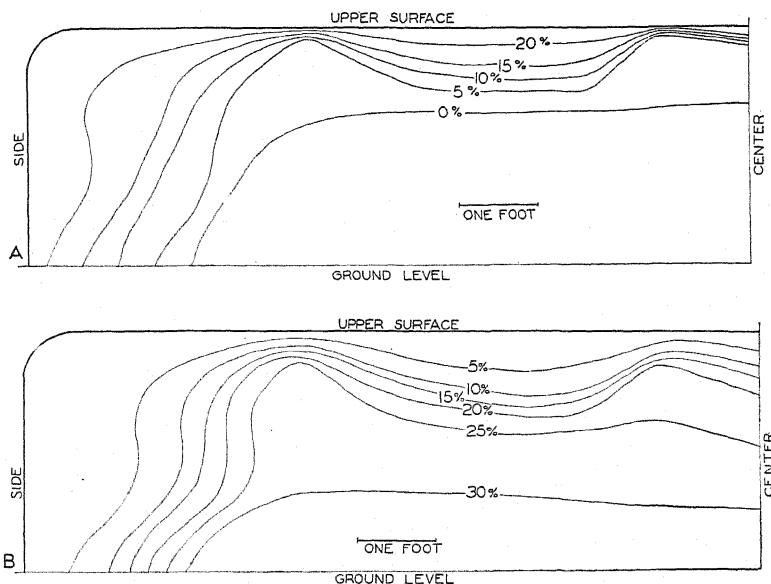
FIGURE 1.—Cross section of a mushroom compost heap from side to center, 12 feet from the end, showing the concentrations of oxygen (*A*) and carbon dioxide (*B*).

Contours showing the concentration of oxygen and carbon dioxide in a typical unventilated heap 3 feet deep are given in figure 1. The dip in the contour lines which appear 5 feet from the side of the heap suggests convection currents.

In most samples in which the oxygen content was more than 1 percent, the sum of the oxygen and carbon dioxide percentages was approximately 21 percent. This, of course, is roughly the percentage of oxygen in the ordinary outside atmosphere, and the constant recurrence of this figure may be taken to indicate that the average respiratory ratio $\left(\dfrac{ccCO_2}{ccO_2}\right)$ of the heterogeneous microbial population of the compost heap approximates unity under aerobic conditions.

The increase in concentration of carbon dioxide seems to reach a limit at approximately 30 percent. The fact that manure decom-

position is arrested in this section is easily observed while the compost heap is being turned, especially during the second turning. At that time the manure in a mound-shaped region in the lower central part of the heap is distinctly "greener" than the remainder of the heap. No attempt was made to determine whether the 30 percent carbon dioxide content is responsible for this retarded fermentation; nor were analyses made of the remaining 70 percent for combustible gases, such as hydrogen and methane, which are known to be generated under similar conditions.

Tests were made to determine the rapidity with which the concentration of carbon dioxide builds up after the manure is turned. As shown in figur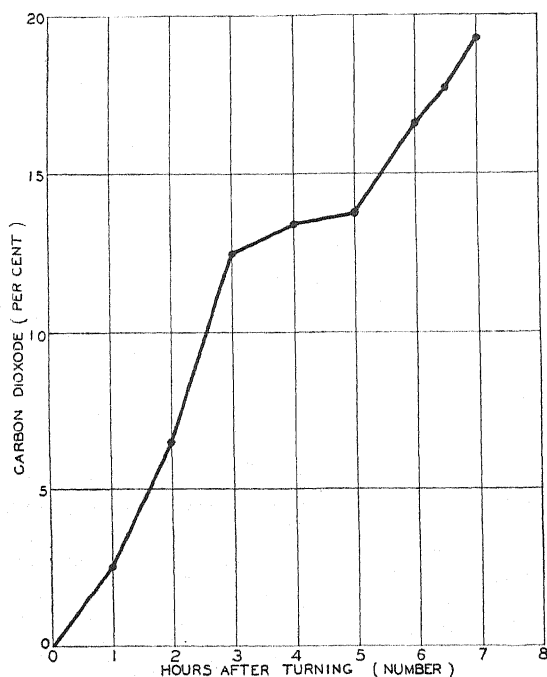e 2, it was found that the carbon dioxide content begins to increase rapidly immediately after turning. In 7 hours the carbon dioxide concentration had reached approximately 20 percent, and no oxygen could be detected.

For several years a few commercial mushroom growers have been placing ventilating tunnels of lattice work under the center of their compost heaps to speed up decomposition. To determine the effect of ventilation of this kind, bench tile was laid on the ground across an experimental compost heap and aspirations were made at different levels above the tile and at different lateral distances from the tiled area. The results of carbon dioxide analyses from these aspirations are given in figures 3 and 4. It should be noted that there is no anaerobic region in any part of the heap above the ventilation tile. Unlike the conditions in ordinary heaps the percentage of oxygen is higher at the bottom than at 1 or 2 feet from the top. The data in figure 4 indicate that the lateral extension of the effect of ventilation tile on aeration is rather limited. In the heap studied anaerobic conditions prevailed at a lateral distance of 2 feet from the tiled area.

FIGURE 2.—Rate of accumulation of carbon dioxide in center of mushroom compost heap after turning.

TESTS FOR TEMPERATURE

Two or three days after the compost was mixed the contours of temperatures were found to be substantially the same in all the flat

heaps studied.   Typical contours are shown in figures 5 to 8.   In general the exterior 3 or 4 inches of the compost heaps varies from slightly above air temperature to above 100° F., depending on the moisture content and the tightness with which the manure is packed. Consistent temperatures begin to be found at a depth of about 6 inches.   At the ground level temperatures are relatively low, usually
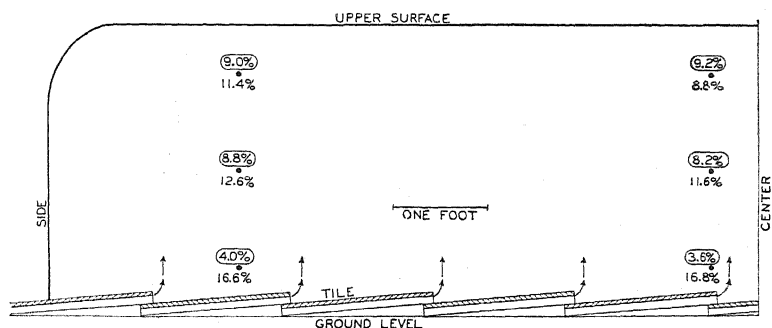

FIGURE 3.—Cross section of one half of compost heap ventilated with greenhouse bench tiles at ground level.   Numbers encircled represent concentrations of carbon dioxide; other numbers represent concentrations of oxygen, at points indicated by dots.   Arrows indicate currents of air.

from 110° to 120° at points within the heap 2 to 4 feet from the side, then dropping as the center of the heap is approached until temperatures of less than 100° may be encountered.   Above the ground there is a similar temperature range.   The low-temperature region forms a low mound in the center of the pile roughly corresponding
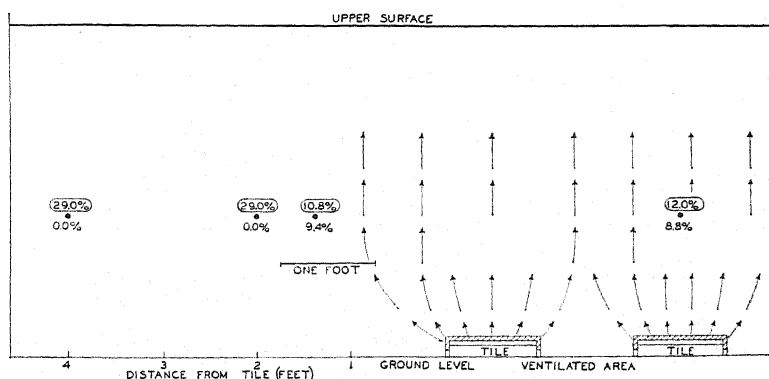
FIGURE 4.—Longitudinal section of portion of compost heap ventilated with greenhouse bench tiles.   Section taken across tiles to show percentages of oxygen and carbon dioxide immediately above, and at different lateral distances from source of air.   Numbers encircled represent percentages of carbon dioxide; other numbers represent percentages of oxygen, taken at points indicated by dots.   Arrows represent currents of air.

to the anaerobic region.   Above this the layers of successively higher temperatures rise in more or less regular strata, following the outlines of the mound.   The hottest portion of the heap occupies the space between the sides of the heap and the central anaerobic mound.   It usually extends from 6 inches to 2 feet down from the top and from 1 to 4 feet in from the sides.   Though usually somewhat oval, it

varies in shape and may contain from 2 to 5 square feet in cross section. In this region, forming a ring like a huge elongated dough-nut about the center of the pile, the temperature is usually in the neighborhood of 170°, although 182° was recorded on one occasion.

In view of the work of numerous investigators on microbial ther-mogenesis (*6, 7, 8*),[2] there can be little doubt that this distribution
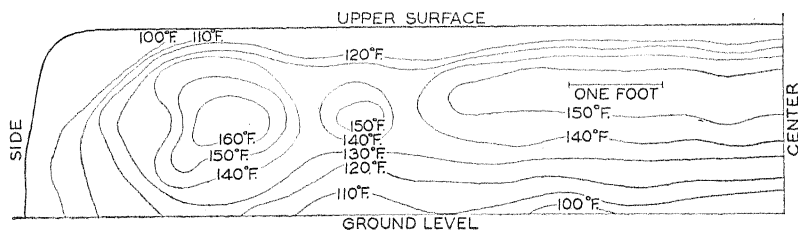
FIGURE 5.—Cross section 6 feet from end of compost heap 2 feet high, showing temperature contours.
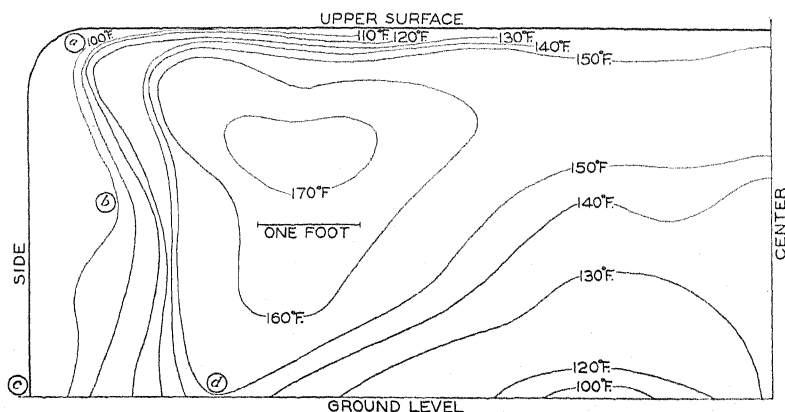
FIGURE 6.—Cross section through compost heap 4 feet high, showing temperature contours. Letters in circles designate points referred to in the text.
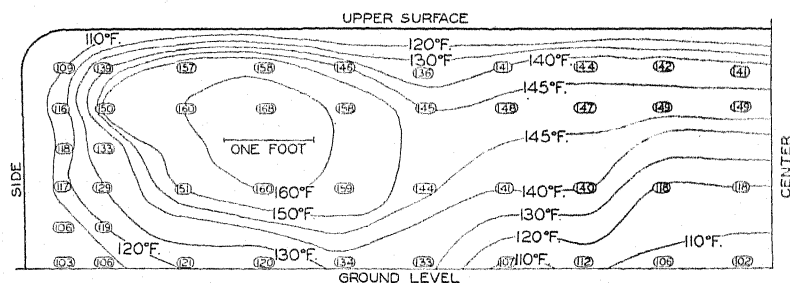
FIGURE 7.—Cross section 6 feet from end of compost heap 3 feet high, showing temperature contours. Ellipses indicate points at which temperatures were taken. Small figures encircled represent tempera-tures at those points.

of temperature is due largely to the thermogenic activities of an active microbial population.

The warm region apparently is favorable for the accumulation of heat because it is well insulated from outside temperatures and at the same time is comparatively well supplied with oxygen. The outer

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[2] Reference is made by number (italic) to Literature Cited, pp. 600 ,601.

layers are cooler because of the lack of insulation from the outside; and the lower central region is cooler because the lack of oxygen retards the microbial activity. The slight extension of the high-temperature region into the adjacent anaerobic region is probably due to the conduction of heat from the aerobic region.

Although moderate aeration seems to be necessary for the production of high temperatures, an excessive current of air through decomposing material may have a cooling effect. This was especially noticeable in heaps of artificial manure made at the Arlington Experiment Farm in the fall of 1930. In these experiments some heaps were made with straw as it came from the straw stack; whereas other heaps were made with the same weight of straw and chemicals, but the straw used had been chopped by means of a corn cutter into 3-inch lengths. There were 12 pairs of these comparable heaps, and in every case during the early part of the composting period the heaps made with short straw were from 40° to 80° F. warmer than those made with long straw. After a few weeks of composting, when the long straw had lost much of its stiffness, the difference was not so noticeable.

Irregularities in the exterior contours of the heaps illustrated in figures 5 and 6 may also have been caused by excessive aeration due to convection currents. In hand-turned piles there is usually more or less "flaking", or stratification, on oblique lines from the center, and the air passes into the heap along these strata. This is probably the cause of the convex contour in figure 6, the warm air rising at *a*, drawing in cool air at *c*, and causing the convexity at *b*. The extension of the hotter regions toward *d* is probably due to oxygen brought in by this fresh air, which is available for the heating of the manure beyond the point where the cooling effect of the excessive aeration is felt. In more uniformly turned piles the contours would be expected to be more regular, as in figure 7.

FIGURE 8.—One half of cross section through center of conical compost heap, showing temperature contours.

In heaps of the same area, but 2 feet in depth, the contour pattern is practically the same on a vertically compressed scale as in the higher heaps, except that the cool central mound tends to occupy a

slightly greater proportion of the heap than is the case in the higher heaps (fig. 6).

A fairly typical contour pattern for conical heaps is shown in figure 8. In this type of heap the cool central region at ground level is relatively small, but the cooling effect of outside air being drawn in at the lower sides influences a relatively far greater region.
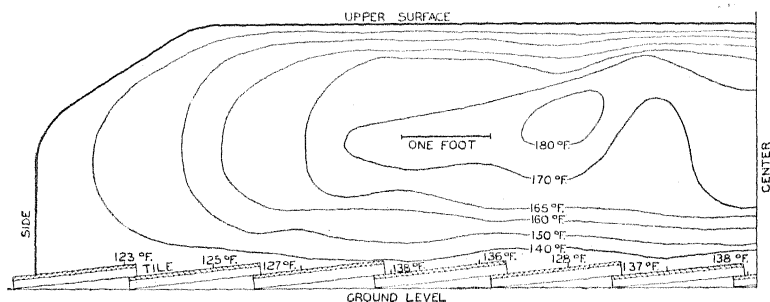
FIGURE 9.—Cross section of compost heap ventilated with greenhouse bench tiles, taken parallel to tiles. Note that whole bottom layer is above 120° F.

In large heaps of the "ridged" type, used in some places, the cross section resembles that of the conical heap, except that the sides are perpendicular to a height of 3 or 4 feet and taper thence to a truncate ridge. These piles are 50 to 60 feet long. In these heaps it would be expected that the contours in cross section would resemble those in figure 8 more or less closely. Because the currents of air have access
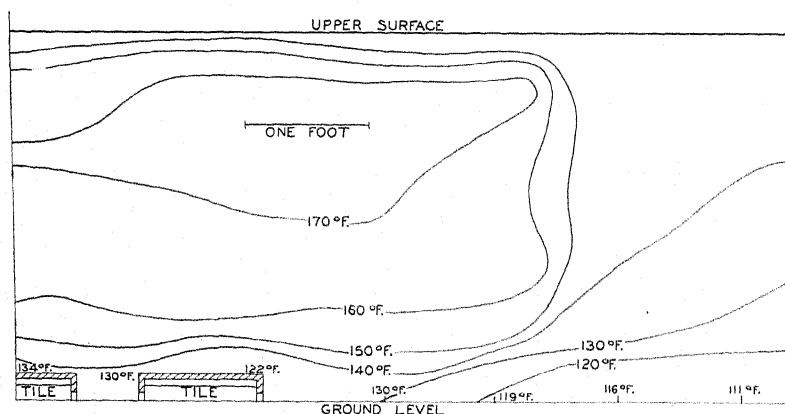
FIGURE 10.—Longitudinal section of compost heap ventilated with greenhouse bench tiles, taken at right angles to tiles.

on only two sides, it is probable that the cooler region at the lower sides will be smaller, and that in the center at ground level larger.

As pointed out in the discussion of aeration, for some time it has been the practice of a few growers to place beneath the composting heaps heavy lattice troughs, or other means of admitting air to the bottom. The effect of this procedure on the temperature contours is

shown in figures 9 and 10.   It will be seen that the temperature at the ground level is raised uniformly to above 120° F.   In the upper strata the high-temperature areas coalesce, so that the temperature contours are flattened out and the temperature of the whole heap is raised and made more nearly uniform.   Undoubtedly this is due to the comparatively uniform distribution of oxygen that was present over the ventilation tiles.   When temperatures were taken in a plane at right angles to the tiles, it was found that the parts of the heap more than a foot away from the tiles at ground level were not much affected.   As would be expected, a foot or so above the tile the heat of the manure extends laterally for a somewhat greater distance. From the data at hand, however, it would seem that the lateral extension of the effect of aerating devices is rather limited.

In order to ascertain how long it would take compost to attain its maximum heat after turning, with its attendant aeration, the bulbs of recording thermometers were placed in various portions of the 4-foot heaps.   In the heaps without tile the temperatures usually reached their maximum in from 18 to 24 hours in the portions of the pile well off the ground but at points at or near ground level continued
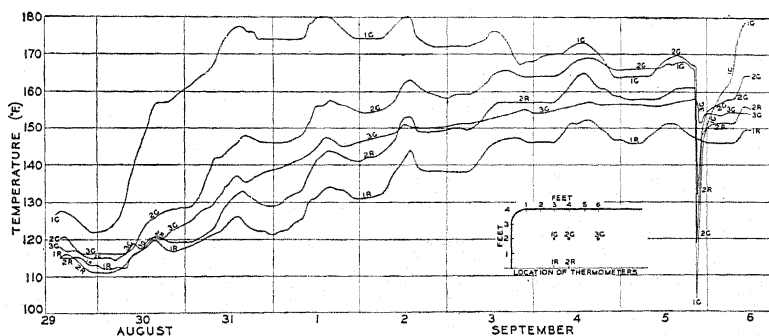
FIGURE 11.—Record of temperatures in different parts of mushroom compost heap after the second turn-ing.   The small diagram indicates the positions of recording thermometers by dots and by the symbols 1–G, 2–G, 1–R, 2–R, etc.

climbing for 144 to 168 hours or more, as shown in figure 11.   In these piles the difference in temperature between the lowest and highest points at the end of a week or 10 days may be as much as 40° F. and was usually in the neighborhood of 20°.   By this time the temperatures are usually running along fairly evenly or dropping slightly.   The influence of external weather conditions is very evident. There is a sharp rise in temperature, even at the bottom of the pile, at about noon on warm days, and a corresponding drop at night.   Rain causes a sharp drop and a nearly equally sharp rise in temperature, affecting the upper portions of the heaps more than those lower down.

In ventilated compost heaps the temperatures rise more slowly, attaining their maximums in from 48 to 72 hours, the lower portion of the heap being the slowest to warm up.   After the maximum temperature has been reached and held for 24 hours or so the tem-perature slowly drops until the next turning, descending perhaps 25°, as shown in figure 12.   The difference between points in the lower and upper portions of the heaps is, as has been pointed out, much less, being only about 8°.   The influence of external weather conditions

is seen in these piles but is very much less evident than in the unventilated ones.

HYDROGEN-ION CONCENTRATIONS

There is a considerable difference in the opinion of different workers on the question of the acidity of the compost. Duggar (*4*) in 1905 stated that manure which has undergone fermentation for a few weeks is usually slightly acid in reaction. This statement was accepted for 20 years and substantiated by Bechman (*2*), who found a reaction of pH 6.4 in manure that had fermented 21 days. On
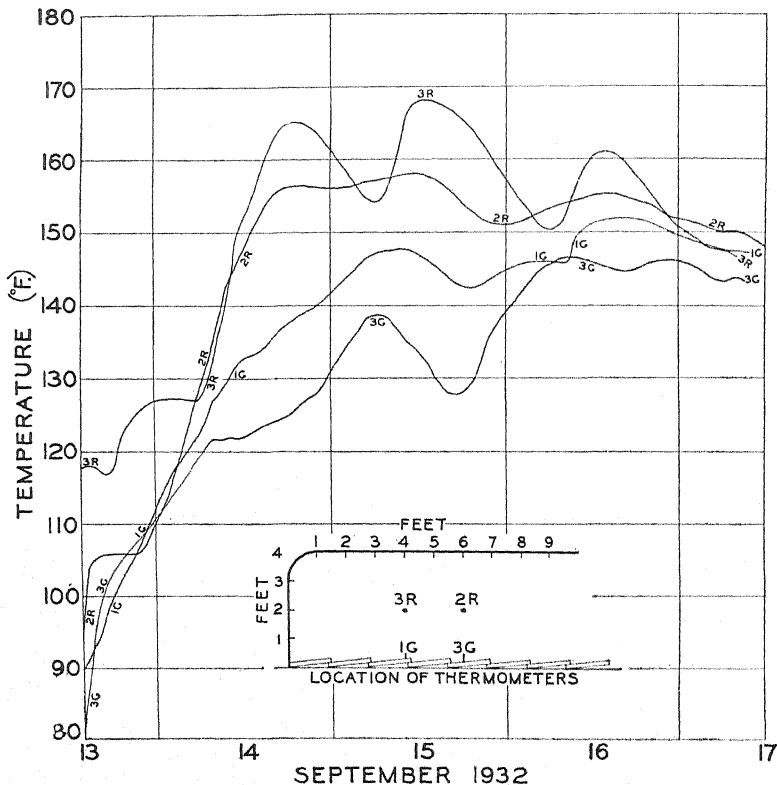
FIGURE 12.—Record of temperatures over tiled portion of mushroom compost heap. The small diagram indicates positions of recording thermometers by dots and by the symbols, 1–G, 3–G, 2–R, and 3–R.

the other hand, Beach (*1*) and Lambert (*9*) found an alkaline reaction in numerous samples of mushroom compost from commercial establishments in eastern Pennsylvania. The pH values recorded in the present study indicate an alkaline condition in well-aerated compost and an acid condition in the parts of the heap composting under anaerobic conditions. It is possible that the discrepancies in the results of different workers can be explained on this basis.

The results of a series of tests of the pH value in different parts of a compost heap are given in figure 13. It is apparent that the outside layer of this heap was largely alkaline or neutral (pH 8.5 to 7.1),

whereas the anaerobic mound at the bottom of the heap was predominantly slightly acid (pH 6.6 to 5.1). The general trend from an alkaline reaction in the outside layers toward a slightly acid reaction in the lower central portion is unmistakable, although there are several notable exceptions. Manure subject to firefang was neutral or alkaline in reaction, and manure over tile ventilation as a rule was more alkaline than manure taken from the bottom of unventilated heaps.

MOISTURE CONTENT

Moisture is one of the most variable factors in a compost heap. In general, mushroom growers attempt to maintain approximately 150 percent of water in the compost on a dry-weight basis. Water is usually added during the process of turning the compost, and in many cases soil is added to the manure to help conserve the moisture. As a result of these practices a moderate moisture content is maintained in most of the compost. On the other hand, there is always a tendency for the compost to dry out excessively on the sides of the heap. This is undoubtedly due to the taking up of moisture by cur-
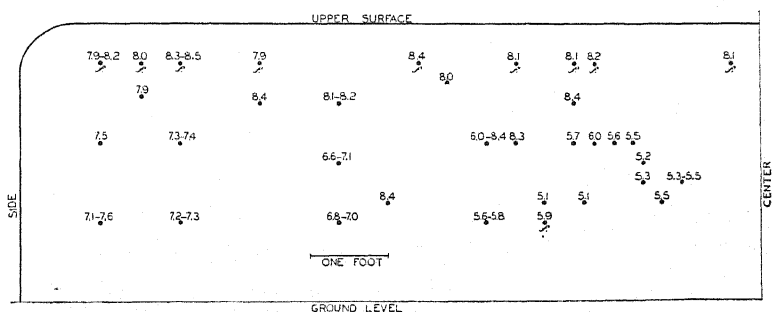
FIGURE 13.—Cross section of compost heap, showing pH values of composting manure, 7 days after second turning, taken at points indicated by dots. The surface layer is alkaline (pH 8.0 to 8.5). The aerated layers on the sides of the heap range from pH 7.1 to 7.6. The wet layer on the bottom center is usually acid (pH 5.1 to 6.6), with occasional alkaline spots (pH 8.0 to 8.4). The firefanged manure (*f*) is aklaline as a rule.

rents of air which are warmed upon entering the heap. A tendency to dry out is noticeable also in the layer of manure in contact with ground-level ventilators. A converse condition is noticeable in the upper 6 inches of the heap. Here the warm air from the lower regions rises to the surface saturated with moisture that condenses when the air reaches the cool outer shell. As a result there is usually a wet layer over the surface of the heap. Toward the end of the composting period there is usually less tendency for the compost to dry out than in the beginning when the straw in the manure is still stiff.

DISCUSSION AND CONCLUSIONS

It is evident from the data presented that there are markedly different conditions of aeration, temperature, acidity, moisture, and rate of decomposition in different parts of ordinary flat mushroom compost heaps and that these conditions are distributed in a regular manner that is fairly consistent for heaps of similar size and shape. Perhaps it would be well to point out here the changes in these con-

ditions to be expected from changes in the size and shape of the heap and some of the implications of these phenomena in the general problem of improving the composting practice for mushroom culture.

Considering aeration first, it has been shown that there is a progressive reduction in oxygen accompanied by an increase in carbon dioxide in the interstices of the manure as the center of a compost heap is approached from the outside, and that 8 hours after turning, anaerobic conditions prevail in regions deeper than 1 foot from the top of the heap and more than 3 feet within the side of the heap. Likewise, it is apparent that when ventilators are run under the center of the heap along the ground the anaerobic condition, high in carbon dioxide, in the lower central part of the heap, is changed to a fairly well aerated one. Vertically this change extends from the ventilators to the top of the heap, but laterally the aeration does not seem to extend more than 2 feet. It is evident from these observations that increases in the height or the width of unventilated compost heaps tend to increase the proportion of manure subject to anaerobic conditions over that subject to aerobic conditions. On the other hand, anaerobic conditions can be entirely eliminated by the use of closely spaced ground ventilators.

The distribution of temperature in the compost heap seems to be dependent on three factors, namely, aeration, conduction, and convection. The highest temperatures (160° to 180° F.) are usually confined to a region 2 to 4 feet within the sides of the heap and 1 to 3 feet from the top. The outer layers are cooler because of the lack of insulation from the outside and the lower central region is cooler because the lack of oxygen retards microbial thermogenesis. Since thermogenesis is retarded by a lack of oxygen, changes in the height or width of the heap can be expected to affect the average temperature in much the same way as aeration is changed. Increases in the height or width of the heap reduce the average temperature by increasing the size of the cool central region, and complementary ventilators placed at the ground level materially raise the average temperature of the heap.

A region containing compost having an acid reaction and having a comparatively slow rate of decomposition corresponds roughly with an anaerobic region, and the proportion of compost subject to these conditions is increased also with increases in the height and width of the heap.

It is a common observation that currents of air passing into the sides of compost heaps or through ventilators at the ground level have a tendency to dry out the compost at the sides of the heap and surrounding the ventilators. Therefore, reducing the width of a heap increases the tendency for it to dry out during composting, and the insertion of ventilators at the ground level has a similar tendency.

It would seem then that within reasonable limits decreasing the lateral dimensions of a compost heap, or reducing the width of the heap as compared to the length, tends to increase the proportion of aerated alkaline compost in the heap, to raise the average temperature, and to increase the tendency of the compost to dry out between turnings. The insertion of ventilators at the ground level has a similar effect. On the other hand, increasing the height of the compost heap, as is frequently done when manure is stored for several weeks,

tends to increase the proportion of anaerobic acid compost in the heap, to decrease the average temperature, and to some extent to reduce the tendency toward drying out.

The foregoing considerations naturally raise the question, What composting conditions are likely to produce the most favorable medium for the growth and yield of mushrooms? At the present time this question cannot be answered in a categorical fashion in terms of size, shape, ventilation, and methods of turning the compost heap. Most commercial growers, when using manure of average texture, make up their heaps about 4 feet high, 20 feet wide, and 40 to 60 feet long. In these heaps about one half of the manure composts under anaerobic conditions; and if it were not for the thorough mixing obtained during the turning process, the lower central part of the heap would take 2 or 3 times as long to decompose as the outer portion. Preliminary experiments and the beneficial effect of the final fermentation in the beds suggest that an aerated condition in the compost heap is preferable to an anaerobic condition provided it can be attained without excessive heating or drying out. Theoretically aeration can be increased by making the heaps narrower or lower, by inserting ventilators at the ground level, or, perhaps preferably, by both increasing the height of the heap and inserting ground-level ventilators. Such changes seem worthy of experimental trial, but it should be recalled that they also may tend toward excessive drying out and overheating and that the beneficial effects of aeration are not well established. The problem can probably best be attacked by a series of semi-empirical yield experiments combined with a study of the microbial and insect population encouraged under different conditions. The large number of factors to be considered and the heterogeneity of stable manure, composting conditions, and conditions during the growth of the crop will make sure progress slow and expensive.

As a working hypothesis it may be assumed that composting conditions which produce a favorable medium for the development of mushrooms probably do so because they encourage the development of a microbial population that is best able to pave the way for the subsequent growth and fructification of mushrooms. Such a hypothesis must take full cognizance of the effect of the staling products of different groups of organisms on mushroom development as well as the action of these organisms in producing changes in the manure favorable to the nutrition of mushrooms under competitive conditions. Interesting facts pertinent to the latter question have been brought to light by the culture studies of Styer (*10, 11*) and Bechman (*2*) and the proximate chemical analyses of Hébert (*5*) and of Waksman and Niessen (*12*).

Raising the temperatures approximately 25° F. at the bottom of the heap by ground-level ventilation suggests interesting possibilities from the standpoint of reducing the introduction of pests into the mushroom houses with the composted manure. If all houses could be properly heated, fumigated during the peak of the heat, and protected thereafter, there would be much less trouble from insect and fungus pests; but at present this ideal is usually not attained, and it is important that the compost be taken into the house as nearly pest-free as possible. In the unventilated heaps a considerable portion of the bottom layer is below 100°, and a still greater proportion below

110°, temperatures that most mushroom pests can survive for some time. It is true that the high carbon dioxide concentration and the lack of oxygen might cause insect and fungus pests to cease activity, but they probably can survive for a long time under those conditions. Temperatures necessary to kill mushroom insects of various species in their various stages have not yet been determined with accuracy but are certainly below 130°. Chapman (*3*) gives 125.6° as the highest authentic record of temperature endured by any insect. In the case of fungus pests the benefits are more uncertain, because some fungus pests are known to withstand temperatures higher than 130°.

SUMMARY

Gas samples taken from all parts of mushroom compost heaps indicate an increase of carbon dioxide and decrease of oxygen toward the lower central part of the heap. In flat heaps 3 feet deep anaerobic conditions are usually found deeper than 1 foot and more than 3 feet from the sides of the heap. Compost in this portion of the heap tends to be acid, while that in well-aerated portions is alkaline or neutral. The highest temperatures (160° to 180° F.) are usually confined to a region 2 to 4 feet from the sides of the heap and 1 to 3 feet from the top. The outer layers are cooler because of the lack of insulation from the outside and the lower central region is cooler because the lack of oxygen retards the microbial activity. At ground level temperatures (100° to 120°) are usually lower than in the higher strata, presumably also because of lack of oxygen. A more uniform distribution of oxygen and wider distribution of the high-temperature region is induced by placing ventilating tiles at ground level. In all probability, conditions such as these influence the suitability of the finished compost for mushroom culture by establishing the trend of the microbial and insect population of the compost heap.

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IRREGULARITIES IN THE INHERITANCE OF THE HAIRY-NECK CHARACTER TRANSPOSED FROM SECALE TO TRITICUM [1]

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INTRODUCTION

A preliminary paper [2] recorded the transfer of the "hairy neck" of rye (*Secale*) to wheat (*Triticum*). This transfer of a definite character is of interest inasmuch as within *Secale* there are certain economic characters, particularly winter hardiness, that are desired in the common wheats. If it can be shown that one character may be successfully transferred from one genus to the other there are good reasons to believe that other characters also may be transferred. This paper deals primarily with the genetic stability and with the behavior of these hairy-neck wheat forms in crosses with different varieties of wheat.

REVIEW OF LITERATURE

Leighty and Taylor [2] and Florell [3] report the isolation of hairy-neck lines from wheat-rye hybrids. Bleier [4] gives a comprehensive review of the work of investigators who have studied phases of the wheat-rye problem. Florell [3] has reviewed studies on the cytology of wheat-rye hybrids.

MATERIALS AND METHODS

Hairy-neck is characterized by the presence of pubescence or hairiness on the peduncle, or that portion of the culm just below the first node of the rachis (fig. 1). In rye plants and in hairy-neck wheatlike segregates from wheat-rye crosses hairiness varies from a few hairs around the apical node of the culm to a dense pubescence extending 3 or more inches below the head.

As reported by Leighty and Taylor,[5] typical wheatlike hairy-neck segregates were selected in 1923 at the Arlington Experiment Farm, near Washington, D.C., from descendants of natural wheat-rye hybrids found in 1918. Ten of these selections were grown originally, but later work was concentrated on three, designated as C, K, and H, which it is believed represent the characteristic behavior of this group of selections.

The three selections are similar to *Triticum vulgare* [6] in their principal spike characters, with the exception of the neck (or peduncle), which is hairy, as shown in figure 2. The plants are not so tall and

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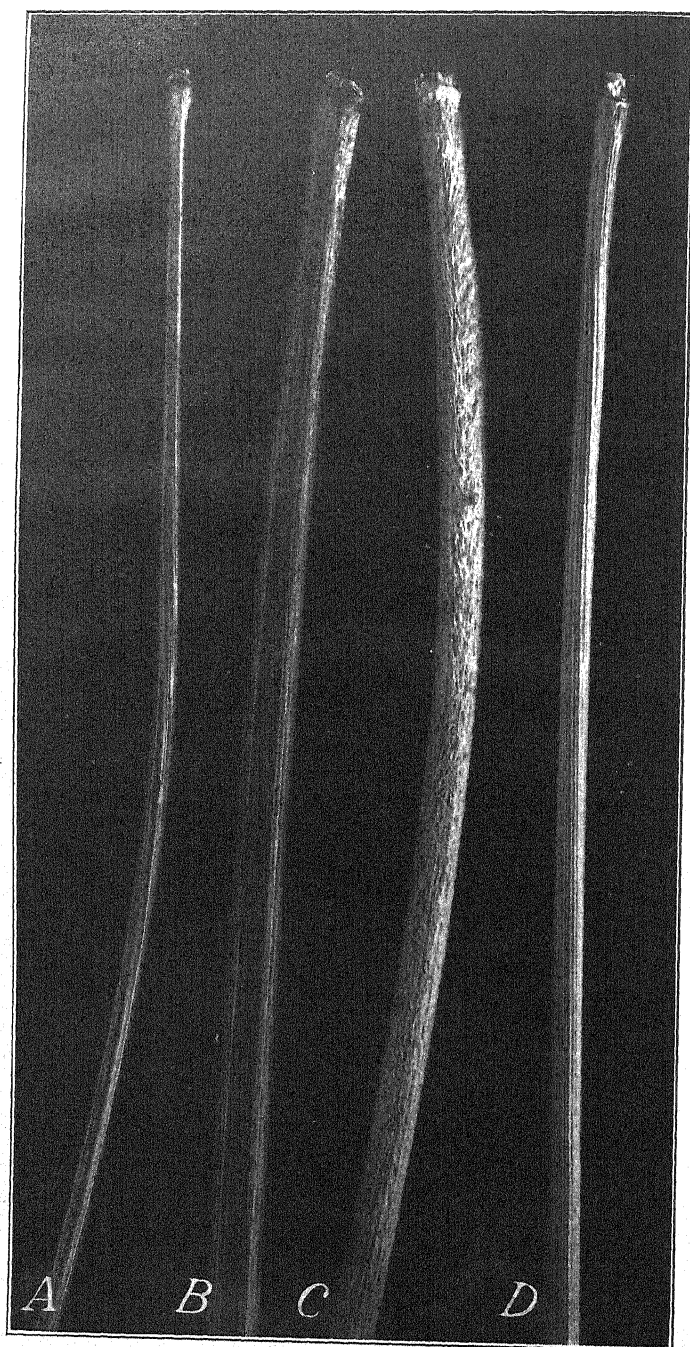
FIGURE 1.—Necks (upper portions of peduncle) of rye, wheat, and two hairy-neck wheatlike selections from wheat-rye hybrids. *A*, Abruzzes rye; *B*, Selection C; *C*, Selection H; *D*, Fulcaster wheat. ×3. *A*, *B*, and *C* are hairy.

are less vigorous than those of wheat, as is usually the case with hairy-neck segregates of wheat-rye crosses. They are more subject to natural crossing than are commercial varieties of wheat, and selfing has been necessary to maintain them. They may be described as follows:

FIGURE 2.—Heads of wheat and three hairy-neck wheatlike selections from wheat-rye hybrids. *A*, Selection K; *B*, Selection H; *C*, Fulcaster wheat; *D*, Selection C. Natural size.

SELECTION C.—Awnless, white glumes, red kernel, with hairs extending one half inch downward from the apical node.

SELECTION K.—Awned, white glumes, red kernel, with hairs extending one half inch downward from the apical node.

SELECTION H.—Awned, white glumes, red kernel, with hairs extending 4 inches or more downward from the apical node. Spike more lax than that of Selection K.

Observations regarding the stability of each of the three selections with respect to the hairy-neck character were made. Each of these selections was crossed with several varieties of soft red winter wheat grown at the Arlington Farm and the progeny were studied in such a way as to determine the nature of the segregation of the hairy-neck character in relation to other characters of the parents. The F_2 populations were grown in spaced nursery rows and the F_3 populations in 5-foot head rows. All hairy-neck plants of each F_2 population which produced sufficient seed for a test were continued in the F_3 generation. Notes on neck character and height were taken in the field. Data on sterility were obtained from the two lower florets of each spikelet of the primary head.

EXPERIMENTAL RESULTS

CONSTANCY OF THE HAIRY-NECK SELECTIONS

The constancy of the hairy-neck character was determined by selfing hairy-neck plants of each of the selections and recording the number of aberrant types appearing in the following generation. The data for Selection C, which is regarded as typical of the hairy-neck segregates, and which has been selfed since 1925, are given in table 1. It will be seen that Selection C does not stand the test of genetic purity expected of a true species. Of 3,818 plants grown during the 8 years, 262, or nearly 7 percent, were different from those of Selection C with respect to the hairy neck.

TABLE 1.—*Constancy of the hairy-neck character in progeny of Selection C selfed for 8 generations*

Year	Total plants	Plants similar to Selection C	Plants differing from Selection C	
			Variant hairy	Variant smooth
	Number	*Percent*	*Percent*	*Percent*
1924	39	92. 3	7. 7	0. 0
1925	675	93. 8	5. 9	. 3
1926	608	94. 7	4. 8	. 5
1927	388	99. 0	1. 0	. 0
1928	435	93. 5	5. 1	1. 4
1929	227	97. 8	1. 8	. 4
1930	423	91. 0	5. 9	3. 1
1931	1, 023	89. 2	9. 6	1. 2
Total or average	3, 818	93. 1	5. 9	1. 0

Two aberrant types appeared, one, designated "variant smooth", indistinguishable from wheat, and the other, designated "variant hairy", almost intermediate with respect to hairy neck between Selection C and wheat. There were approximately 1 percent of the former and 6 percent of the latter. Variant smooth is easily distinguished from Selection C because it is smooth-necked and taller. Variant hairy is from 4 to 6 inches taller than Selection C when grown under favorable conditions, but the difference in height may not be apparent under unfavorable conditions. Partly for this reason it is not so readily recognized. However, there is good reason to believe that none of the conclusions arrived at herein is invalidated by errors of classification.

During the 8 years Selection C never behaved as a pure line. Variant hairy necks were found every year and variant smooth necks in 6 of the 8 years. The greatest irregularity occurred in 1931, when 110 plants of a population of 1,023 plants, or 10.8 percent, were variants. The least variation occurred in 1927, when only 1 percent were variants.

Additional data regarding the constancy of Selection C and the breeding behavior of the variants selected from it were obtained by growing in 1930 a selfed plant of Selection C and selfing the progeny and growing them in 1931. The pertinent data are presented in table 2.

The progeny of the single selfed plant in 1930 were classified as 50 similar to Selection C, 1 variant hairy, and 2 variant smooth. Only 49 of the 50 plants of Selection C indicated in table 2 were grown in 1931, 1 failing to produce sufficient seed. Each of the 49 plant rows supported the 1930 classifications, breeding typical Selection C with 7.3 percent variants. The 2 variant smooth-neck plants bred smooth, and the variant hairy-neck plant segregated in the ratio of 3 smooth to 1 hairy.

TABLE 2.—*Breeding behavior of the hairy-neck character in the progeny of a plant of Selection C during 2 generations of selfing, 1930 and 1931*

Progeny from selfed plant, 1930		Progeny from second generation of selfing, 1931			
Type	Total plants	Total plants	Type of plant		
			Selection C	Variant hairy	Variant smooth
	Number	*Number*	*Percent*	*Percent*	*Percent*
Selection C	50	384	92.7	7.0	0.3
Variant hairy	1	8	.0	25.0	75.0
Variant smooth	2	26	.0	.0	100.0

The percentage of smooth-neck plants in this particular line of Selection C in 1930 and 1931 was somewhat less (0.7 percent), and the proportion of variant hairy plants slightly more (6.4 percent) than the average shown in table 1.

During this study of the inconstancy of Selection C 30 variant hairy-neck plants were grown in head or plant rows. These produced 1,388 plants of which 321, or 23.1 percent, were hairy-necked and 76.9 percent smooth-necked, thus approximating the results, presented later, of crosses between Selection C and wheat.

The average proportion of smooth-neck plants appearing in Selection C, that is, about 1 percent, may be explained by assuming a simultaneous loss of the hairy-neck factor in 10 percent of the pollen cells and egg cells. The expected proportion of smooth-neck, variant hairy-neck (heterozygous), and Selection C types is then given by the expansion of the binomial $(1+9)^2$. The fact that the smooth-neck plants bred true and the hairy-neck plants bred like F_1 hybrids is in accord with this hypothesis. However, the average proportion of variant hairy-neck plants, approximately 6 percent, is only about one third of the number to be expected on this basis. It seems necessary to assume also differential functioning or vigor of the two types of gametes

or zygotes, or it is possible that the loss of the hairy-neck factor may occur in a somatic division in the development of the primordium for the flowers of a spike.

INHERITANCE IN CROSSES OF HAIRY-NECK SELECTIONS × WHEAT

Hybridizing the hairy-neck selections with common wheat may be expected to give further information as to the genetic irregularity of the hairy-neck character and its relation to the inheritance of certain common wheat characters. In 1923 and later, selections which from phenotypic indications were pure for the hairy-neck character of the C, K, and H selections, were crossed with common wheat varieties. The varieties chosen differed in such head characters as awnlessness and awnedness, red and white glume color, and smoothness and pubescence of glumes. The segregation of the common allelomorphs permitted observation as to the effect of an intergeneric character on their behavior.

The F_1 hybrids conformed in the more common spike characters to what would be expected in crosses of wheat varieties; that is, there was expressed the incomplete dominance of awnlessness, red glume color, and pubescent chaff. The hairy-neck character was dominant, but the hairs did not extend downward so far as in the parental selection, and the density of the hairiness was decidedly reduced. The F_1 heads appeared fully fertile and were selfed.

The hairy-neck selections were crossed with one or more of the varieties of *Triticum vulgare*, namely, Brown Fife,[7] Purplestraw, Fulcaster, Nittany, Poole, and Fultz. All except Fulcaster and Nittany are awnless, and all except Brown Fife and Poole have glabrous white glumes. Brown Fife has pubescent red glumes, and Poole has glabrous red glumes.

Glume color developed poorly, and segregates from this character were not classified, although it was evident that hairy neck was present in both the red- and white-glume segregates of the F_2 generation. The number of F_1 plants secured in each case, the number of F_2 plants that were grown, and the segregation of the latter with respect to presence of awns, pubescence of glumes, and hairy necks are shown in table 3.

The segregation with respect to awns and pubescence is what would be expected when varieties of *Triticum vulgare* possessing these characters are crossed. In the six crosses involving awn segregation, the fully awned recessive constitutes 24.4 percent of the total number of plants that were grown. In the single cross (Selection C × Brown Fife) involving pubescent and glabrous glumes, 24.1 percent of the plants had glabrous glumes. On the other hand, the segregation with respect to the hairy-neck character was quite irregular. In the five crosses involving Selection C the percentage of hairy-neck plants ranged from 17.7 to 29.2 and averaged 25. In the four crosses involving Selection K, the percentage of hairy-neck plants ranged from 30.6 to 48. 2, with an average of 36.2. There were two crosses involving Selection H. In these the percentages of hairy-neck plants were 61 and 63.4, respectively, averaging 62.9.

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[7] The name "Brown Fife" was given in 1922 to a strain of wheat formerly grown as Jones Winter Fife. In habit of growth and morphological characters it is somewhat similar to Grandprize.

TABLE 3.—*Segregation in the F_2 generation from crosses of 3 hairy-neck selections with varieties of common wheat at the Arlington Experiment Farm, Rosslyn, Va.*

Cross	F_1 plants	F_2 plants	F_2 plants of indicated class					
			Awnless				Awned (glabrous)	
			Pubescent		Glabrous			
			Hairy	Smooth	Hairy	Smooth	Hairy	Smooth
	Number	*Number*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Selection C × Brown Fife	1	220	12.3	63.6	5.5	18.6	0.0	0.0
Selection C × Purplestraw	2	1,365	.0	.0	26.4	73.6	.0	.0
Selection C × Fulcaster	1	168	.0	.0	22.6	57.7	6.6	13.1
Nittany × Selection C	12	343	.0	.0	15.2	58.6	5.8	20.4
Selection C × Poole	1	171	.0	.0	26.9	73.1	.0	.0
Selection K × Purplestraw	4	486	.0	.0	37.7	39.5	10.5	12.3
Selection K × Fultz	13	281	.0	.0	23.8	50.2	8.2	17.8
Fulcaster × Selection K	1	191	.0	.0	.0	.0	38.7	61.3
Poole × Selection K	8	950	.0	.0	21.9	52.1	8.7	17.3
Selection H × Fultz	1	100	.0	.0	51.0	27.0	10.0	12.0
Selection H × Fulcaster	9	383	.0	.0	.0	.0	63.4	36.6

In none of the crosses involving Selections K and H do the ratios conform to simple Mendelian inheritance. The average results for Selection C agree exactly with expectations for a monohybrid, except that hairy neck behaves as the recessive, whereas in the F_1 this character was dominant. The breeding behavior of the F_1 of Selection C × wheat is similar to that of the hairy-neck variants.

There is no indication of linkage of the hairy-neck character with either of the other characters studied except in the Selection H × Fultz cross, in which the proportion of hairy necks in a small population is approximately twice as great for the awnless segregates as for the awned.

A number of the crosses were continued into the F_3 generation. Some of these were space-planted, but the greater number were grown in 5-foot head rows. In some cases all the plants from the F_2 rows were grown, whereas in others only the hairy-neck plants were grown. The progeny of 388 smooth-neck F_2 plants were grown and all bred smooth neck. The data for the hairy-neck plants are presented in table 4.

Of the 125 F_3 lines grown from hairy-neck F_2 plants of the two crosses of Selection C, only 3, or 2.4 percent, were homozygous. If the hairy-neck character were a simple recessive, 33.3 percent should be homozygous.

Of the 83 hairy-neck F_2 plants of the cross Selection K × Fultz grown in the F_3, approximately 11 percent were homozygous hairy neck. However, of the 76 F_3 lines of the cross Selection K × Purplestraw 25 percent were homozygous. In the F_2 of Selection K × Purplestraw approximately 50 percent of the plants were hairy neck as compared to 32 percent in the cross Selection K × Fultz. In the former cross the F_2 homozygous hairy-neck plants appeared more than twice as often as in the latter cross.

TABLE 4.—*Breeding behavior of hairy-neck F_2 plants from crosses of hairy-neck selections × wheat*

Cross	F_3 lines		
	Number	Heterozygous hairy neck	Homozygous hairy neck
		Percent	*Percent*
Selection C × Purplestraw	71	98.6	1.4
Nittany × Selection C	54	96.3	3.7
Selection K × Fultz	83	89.2	10.8
Selection K × Purplestraw	76	75.0	25.0
Selection H × Fultz	54	81.5	18.5

The F_1 of Selection K × Fultz was grown in 1925 and the F_1 of Selection K × Purplestraw in 1928. The difference in percentage of homozygosity of the two crosses is believed to be due to differences in the two seasons, inasmuch as 30 of the segregating F_3 lines of Selection K × Purplestraw, involving 545 plants, were space-planted and 25.5 percent of the plants were hairy as compared to approximately 48.1 percent in the F_2 generation. The higher percentage of hairy-neck plants in the F_2 generation of this cross as compared to that of the other three crosses of Selection K × wheat, and the comparatively high percentage of F_3 homozygous hairy-neck lines, indicate conditions unusually favorable for either the formation or the functioning or both of the hairy-neck gametes of the F_1 plants.

Of the 54 F_3 lines of Selection H × Fultz, 18.5 percent were homozygous hairy-neck. Selection H crosses produced approximately 61 percent of hairy-neck plants in the F_2; that is, the hairy-neck character behaved as dominant. However, the F_3 test clearly shows a lethal factor operating to eliminate the homozygous hairy-neck type.

EFFECT OF HAIRY NECK ON PLANT CHARACTERS

The supposition of a lethal effect of the hairy-neck factor suggested the desirability of a study of sterility, seed germination, height of plant, and tillering of the crosses, especially with respect to the smooth-neck and hairy-neck segregates.

STERILITY

The percentages of sterile florets of the hairy-neck selections and of the F_1 hybrids between these and certain varieties of wheat are shown in table 5.

TABLE 5.—*Floret sterility of hairy-neck selections and of F_1 hybrids of hairy-neck selections × wheat*

Selection or F_1 hybrid	Florets	Seeds	Sterile florets
	Number	*Number*	*Percent*
Selection C	1,190	789	33.7
Nittany × Selection C	369	321	13.0
Selection C × Purplestraw	1,356	1,249	7.9
Selection K	282	159	43.6
Selection K × Fultz	536	487	9.1
Selection H	204	160	21.6
Selection H × Fultz	106	102	3.8

In Selection C, 33.7 percent of the florets were sterile, and in the F_1 hybrid of Selection C × wheat and its reciprocal, approximately 10 percent of the florets were sterile. This is about the average sterility for wheat. The average sterility of Selection K was 43.6 percent and of Selection H, 21.6 percent. The F_1 hybrids with wheat in each case were as fertile as would be expected for wheat, the sterility of Selection H × wheat being only 3.8 percent. Selection H has the highest fertility of the three selections and the same relation exists between the F_1 hybrids with wheat. It is pertinent in this connection to note that in the F_2 generation 61 percent of the plants of this cross had hairy necks as compared with 25 and 36 percent in Selections C and K, respectively (table 3). In Selection H, hairiness extends 4 inches down the peduncle as compared to approximately one half inch in the other two selections; that is, the degree of hairiness in these selections was not positively correlated with reduction of fertility as might be expected.

SEED GERMINATION

Seed of the F_1 plants of wheat × Selection C was planted and allowed to mature. Approximately 85 percent of the planted seeds matured plants. A similar study was made of Selection K × wheat from F_2
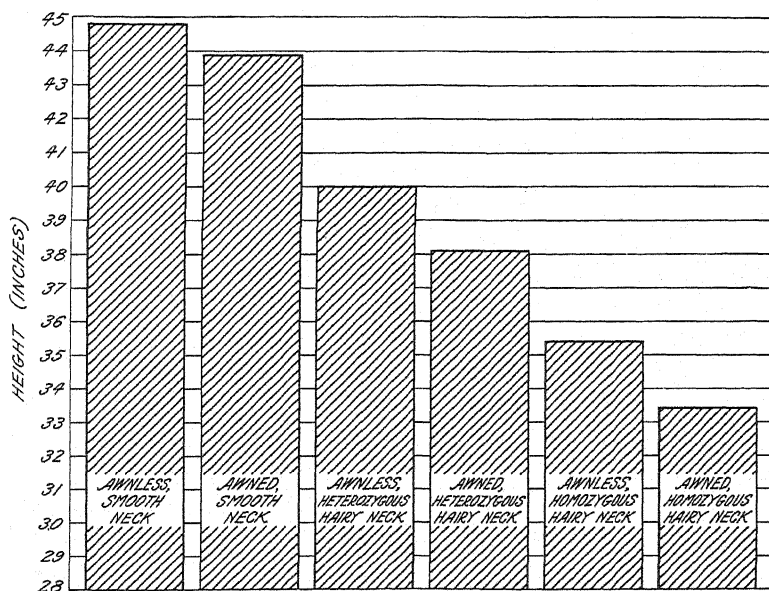
FIGURE 3.—Height of plants of different awn and neck types in the F_3 generation of the cross Selection K × wheat.

seed, as no F_1 seed was available. Conditions for germination were poor, and only 49 percent of the seed of segregating hairy-neck lines and a like percentage of homozygous hairy-neck lines matured plants. A similar planting of smooth-neck seed from the same cross matured only 48 percent of plants. In neither case is there any evidence of differential zygotic lethals.

HEIGHT OF PLANT

The height of a large number of plants in the F_2 and F_3 generations was measured, and the data are presented in table 6. In each of the 17 possible comparisons of the hairy-neck with the smooth-neck classes the hairy-neck plants were significantly shorter, in most cases materially so. The average height of the smooth-neck plants was 46.9 inches as compared to 41.4 inches for the hairy-neck plants. Furthermore, the homozygous hairy-neck F_3 lines were approximately from 2 to 5 inches shorter than those segregating for hairy neck. The comparative height differences in the classes obtained from the cross of Selection K × Fultz are shown graphically in figure 3. No significant differences were found between the height of the plants as a result of the presence or absence of awns.

The commonly cultivated rye varieties have hairy necks. A few strains of smooth-neck rye have been bred, the height of which is no greater than that of their hairy-neck relatives. It is probable, therefore, that the hairy-neck character in the presence of the rye-chromosome set does not adversely affect the height of the plant. The average height of the rye varieties grown at the Arlington Experiment Farm varies from 62 to 65 inches as compared with 46 and 54 inches in the wheat varieties.

TABLE 6.—*Average height of hairy-neck and smooth-neck plants in heterozygous and homozygous hairy-neck lines from hybrids of hairy-neck selections × wheat*

Class and hybrid	Gener-ation	Plants	Average height of plants of indicated class			
			Awnless smooth	Awnless hairy	Awned smooth	Awned hairy
HETEROZYGOUS		*Number*	*Inches*	*Inches*	*Inches*	*Inches*
Selection K × Fultz	F_2	281	47.5±0.42	40.7±0.39	47.7±0.40	38.7±0.85
Do	F_3	1,466	44.8± .36	40.0± .38	43.9± .47	38.1± .57
Selection C × Purplestraw	F_2	1,172	44.1± .39	39.6± .45		
Selection H × Fultz	F_2	90	47.5± .55	42.8± .66	47.6± .69	40.9±1.83
Do	F_3	443	49.1± .14	45.2± .16	49.8± .11	46.9± .16
HOMOZYGOUS LINES						
Awnless hairy (Selection C × Purple-straw)	F_3			36.1± .44		
Awned hairy (Selection K × Fultz)	F_3					33.4± .80
Awnless hairy (Selection K × Fultz)	F_3			35.4± .97		
Awned hairy (Selection H × Fultz)	F_3					44.8± .80
Awnless hairy (Selection H × Fultz)	F_3			43.2± .72		

TILLERING

Data on tillering were obtained for individual plants in the F_2 generation of the two crosses Selection K × Fultz and Selection H × Fultz. The former was grown on more productive land than the latter. In all cases the smooth-neck plants tillered more than did the hairy-neck plants (table 7). The differences between awned and awnless plants were not statistically significant.

TABLE 7.—*Average number of tillers per plant in F₂ classes of hairy-neck selections × wheat crosses*

Cross	Average tillers per plant in indicated F₂ class					
	Awnless			Awned		
	Hairy	Smooth	Difference	Hairy	Smooth	Difference
	Number	*Number*	*Number*	*Number*	*Number*	*Number*
Selection K × Fultz	6.7±0.39	8.5±0.46	1.8±0.60	6.6±0.58	9.5±0.46	2.9±0.74
Selection H × Fultz	3.7± .12	5.3± .30	1.6± .32	3.4± .37	4.5± .49	1.1± .61

BACK-CROSSING F₁ HYBRIDS WITH WHEAT

Since no evidence of zygotic elimination was obtained it seemed desirable to resort to back-crossing to test for comparative functioning of male and female gametes carrying the smooth-neck and hairy-neck factors. This was done by reciprocally crossing the F₁ hybrids with wheat, only the F₁ hybrids of Selection C and Selection K being used. The resulting progeny were then classified with respect to the hairy-neck character. Errors due to self-pollinated plants were eliminated by the selection of a wheat variety in which selfing could be detected. The data are presented in table 8.

The female gametes of Selection K × Fultz, fertilized by wheat pollen, produced plants of which 16.9 percent had hairy necks, whereas the male gametes of the same hybrid, fertilizing wheat egg cells, produced but 9.0 percent of hairy-neck plants. Similarly, the female gametes of the F₁ of Selection C × wheat (Purplestraw and Nittany), fertilized by wheat pollen, produced 13.2 percent of hairy-neck plants, whereas the male gametes of the same hybrid, fertilizing wheat egg cells, produced 8.9 percent of hairy-neck plants. In all back crosses except one a larger percentage of hairy-neck plants was produced when the F₁ hybrid was used as the female parent. However, neither the functional male nor female gametes carried the hairy-neck character in the expected frequency, since in back-crossing experiments such as these the hairy-neck and smooth-neck gametes should occur in equal numbers.

TABLE 8.—*Hairy-neck and smooth-neck plants resulting from reciprocal back-crossing of the F₁ hybrid of hairy-neck selections × wheat with wheat*

F₁ hybrid	Year	F₁ hybrid as the female produced—			F₁ hybrid as the male produced—		
		Smooth-neck plants	Hairy-neck plants		Smooth-neck plants	Hairy-neck plants	
		Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
Selection K × Fultz	1925	23	6	20.7	17	3	15.0
Do	1926	46	8	14.8	54	4	6.9
Total or percent		69	14	16.9	71	7	9.0
Selection C × Purplestraw	1926	34	6	15.0	46	3	6.1
Nittany × Selection C	1929	18	5	21.7	6	0	0
Selection C × Purplestraw	1930	93	11	10.6	92	11	10.7
Total or percent		145	22	13.2	144	14	8.9

Theoretically, there should be agreement among the F_2 segregation, the F_3 family behavior (that is, whether homozygous or heterozygous for the neck character), and the results from the back crosses. The latter indicated that approximately 16.9 percent of the functional eggs and 9.0 percent of the male cells of Selection K $\times$ Fultz (table 8) carry the hairy-neck factor. Assuming the same gametic frequency $(1 + 5)$ $(1 + 10)$ and the same functioning in the selfed F_1 hairy neck $\times$ wheat hybrids, the F_2 population should contain 24.2 percent of hairy-neck plants, and approximately 6.3 percent of these should be homozygous in F_3. Actually, 32 percent of the F_2 plants were hairy (table 3), and 10.8 percent of the F_3 lines were homozygous (table 4).

In Selection C $\times$ wheat slightly more than 13 percent of the egg cells and about 9 percent of the male cells carried the hairy-neck character. On this basis the F_2 population from the selfed F_1 hybrids should be approximately 21.0 percent hairy neck, and 5.7 percent of these should be homozygous. The percentage of the hairy-neck plants actually observed in the F_2 generation of the same crosses (Purplestraw and Nittany) was 25.3 (table 3), and 2.4 percent of these bred true (table 4).

The agreement between the data of the different experiments is perhaps as close as should be expected, considering the small populations obtained from the back crosses and the apparent irregularity in genetic expression due to environment.

DIFFERENTIAL FUNCTIONING OF POLLEN CELLS

The low percentage of functional gametes carrying the hairy-neck factor, as shown in the reciprocal back crosses of the F_1 hybrids of hairy-neck selections $\times$ wheat with wheat, would indicate elimination of the hairy-neck character at meiosis or at fertilization. The high percentage of fertility in the F_1 hybrids of hairy-neck selections $\times$ wheat favors the assumption that functional female gametes carrying the hairy-neck factor are not formed in the expected frequency. The apparent differential functioning of the male gamete of the F_1 hybrids carrying the hairy-neck and smooth-neck characters (table 8) may, however, be due to a growth differential between the two types rather than to nonformation of pollen cells carrying the hairy-neck factor. Experiments were therefore made with mixtures of pollen of Selection C and pollen of three varieties of common wheat, namely, Nittany, Dixie, and Red Rock. Heads of the wheat or of Selection C were emasculated and at the proper time were pollinated with a pollen mixture or first with pollen of Selection C and then with pollen of the wheat variety; in the latter case the interval between the two pollinations averaged about 2 minutes. The pollen mixture was composed of the contents of the same number of anthers of Selection C and of the wheat variety. The anthers of Selection C are larger than those of the wheat varieties used.

When wheat was the female, the progenies were grown and classified as smooth neck or hairy neck, the results showing which pollen grain functioned. When Selection C was the female, glume color or awn contrast of the following progenies showed when the wheat pollen grain functioned, except in Dixie, when the plants were carried to the F_2 generation to identify them. Results of the pollinations are shown in table 9.

TABLE 9.—*Comparative functioning of pollen of hairy-neck selection C and wheat varieties in pollen-mixture and double-pollination experiments*

Female parent	Year	Pollen source	Plants of indicated type resulting from pollination			Flowers fertilized by pollen carrying hairy neck
			Wheat	Selection C	Hybrid	
			Number	*Number*	*Number*	*Percent*
Nittany	1928	Mixture Nittany and Selection C	10	--------	1	--------
Dixie	1928	Mixture Dixie and Selection C	11	--------	0	--------
Red Rock	1929	Mixture Red Rock and Selection C	17	--------	2	--------
Nittany	1928	Selection C and Nittany	11	--------	3	--------
Dixie	1928	Selection C and Dixie	12	--------	1	--------
Red Rock	1929	Selection C and Red Rock	42	--------	5	--------
Total			103	--------	12	10.4
Selection C	1928	Mixture Dixie and Selection C	--------	2	22	--------
Do	1928	Selection C and Nittany	--------	2	14	--------
Do		Selection C and Dixie	--------	0	7	--------
Do	1929	Selection C and Red Rock	--------	1	8	--------
Total			--------	5	51	8.9

One hundred and fifteen plants resulted from pollinating the common wheat varieties with pollen from the two sources. Only 12, or 10.4 percent, were hairy-neck hybrids, the remainder being selfs. When the wheats were pollinated with the mixture the percentage of hybrids was 7.3, and when pollinated first with pollen of Selection C and then selfed the percentage of hairy-neck hybrids increased to 12.2, possibly indicating an effect due to rate of pollen germination or of pollen-tube growth.

Fifty-six plants were secured in the experiments in which Selection C was the female. Fifty-one, or 91 percent, proved to be hybrids and only 5, or 9 percent, were selfs. Approximately the same number of flowers of wheat and of Selection C were pollinated in these experiments, and the fewer seeds and plants obtained indicates again the sterility of Selection C as compared with that of wheat. These results suggest that the pollen cells of Selection C which carry the hairy-neck factor are less viable or that the pollen tube grows more slowly than that of normal wheat. Poor functioning of pollen cells carrying the hairy-neck factor appears at least as probable as nonformation at meiosis in the F_1 hybrid. This is further supported by the agreement between the results from back-crossing the F_1 of Selection C × wheat with wheat (table 8) and the results from pollinating wheat and Selection C with the pollen mixture. Wheat fertilized with pollen from the F_1 hybrid (Selection C × wheat) produced 8.9 percent of hairy-neck plants, whereas wheat fertilized with a mixture of pollen from Selection C and wheat produced 10.4 percent of hairy-neck plants; and Selection C fertilized by a mixture of pollen from Selection C and wheat produced 8.9 percent of selfed hairy-neck plants (table 9).

DISCUSSION

The genetic behavior of the hairy-neck wheatlike selections isolated from wheat-rye hybrids shows that the addition of the rye character results in an unbalanced type. Hairy-neck is a tangible rye character transposed to types that are apparently otherwise *Triticum vulgare.* A preliminary cytological examination of one of the hairy-neck plants

made by Florell [8] showed 44 chromosomes in the root tips as compared to 42 for *T. vulgare*. Inasmuch as the hairy-neck plants are not constant, their chromosomal constitution seems to be better represented by the quantitative expression $2n+2$ rather than $2n$, indicating in this case no homologue in the wheat complement for the rye chromosome. Blakeslee [9] uses the formula $2n+2$ for one of his Globe mutants in *Datura* where the unbalance was of a simple tetrasomic type.

To explain the genetic behavior of the hairy-neck character it may be assumed that the $2n+2$ hairy-neck plants normally produce $n+1$ gametes but that occasionally in male and female gametogenesis the rye chromosome is lost, giving a gamete of n constitution. The fertilization of $n+1 \times n$ gametes results in a zygote similar in later behavior to the cross hairy-neck selection $\times$ wheat, whereas the mating of $n \times n$ gives a zygote which produces a plant indistinguishable from *Triticum vulgare*.

The chromosome number of the F_1 hybrid hairy-neck selection $\times$ *Triticum vulgare*, and also of the variant hairy type, would be $2n+1$ and the plants would be of the hairy-neck type as the character is dominant over the smooth neck. In gametogenesis and fertilization, irregularities in the functioning of $n+1$ and n gametes apparently occur, as the F_2 segregation often shows the hairy character as recessive, and results from the back crosses indicate that from 13 to 17 percent of the functional egg cells and approximately 9 percent of the functional pollen grains carry the hairy character. Furthermore, F_3 lines homozygous for hairy neck do not appear in the expected frequency even for a recessive character. Reduced height and tillering and varying degrees of sterility in the plants with hairy neck as compared to those with smooth neck, in addition to the genetic irregularities, support the belief that there is incompatibility between the wheat and rye complexes and that the reaction is unfavorable both to the normal productiveness of the plant and to its constancy in breeding. Whether the addition or substitution of other rye chromosomes in the wheat complement would react similarly is questionable. Wheat-rye hybrids carrying all the chromosomes in both wheat and rye, $2n=56$, have been produced,[10] but the economic value of such plants has not seemed particularly promising in the United States. Wheat breeders in general are interested in obtaining a definitely *T. vulgare* type with certain desired rye characters rather than a type intermediate between the two genera.

SUMMARY AND CONCLUSIONS

Complete genetic balance has not been obtained in three hairy-neck selections of wheat $\times$ rye crosses designated as Selection C, Selection K, and Selection H. In spite of continuous selfing, approximately 1 percent of the plants of Selection C had smooth necks and bred true and about 6 percent had hairy necks and bred in the same manner as the F_1 hybrids.

The observed proportion of smooth-neck plants may be explained by assuming a simultaneous loss of the hairy-neck factor in 10 per-

[8] Letter addressed to J. W. Taylor by V. H. Florell, Feb. 28, 1931.
[9] BLAKESLEE, A. F. VARIATIONS IN DATURA DUE TO CHROMOSOME NUMBER. Amer. Nat. 56: 16-31, illus. 1922.
[10] LEVITSKY, G. A., and BENETZKAIA, G. K. CYTOLOGICAL INVESTIGATIONS OF CONSTANT INTERMEDIATE RYE-WHEAT HYBRIDS. (PRELIMINARY COMMUNICATION.) U.S.S.R. Cong. Genet., Plant and Animal Breeding, Proc. 2: 345-352, illus. 1930. [In Russian. English Summary, pp. 350-352.]

cent of the pollen cells and egg cells, but the observed proportion of hybrid hairy-neck plants has been only about one third of the number to be expected on the basis of this explanation. It seems necessary to assume also differential functioning or vigor of the two types of gametes or of the zygotes, or possibly loss of the hairy-neck character in somatic mitosis.

In crosses between the three selections and several varieties of wheat the hairy-neck character appeared to be dominant, but in later generations it behaved as a recessive or in an irregular manner.

There appeared to be no linkage of the hairy-neck character with glume color, with condition of glumes in regard to pubescence, or with condition of heads in regard to awns.

In studies of sterility it was found that Selection C, Selection H, and Selection K were materially less fertile than wheat, but that the F_1 hybrids were approximately as fertile as wheat. There was no observable inverse relation between degree of hairiness and sterility, as might be expected; Selection H, which had more hair on the necks than the others, was the most fertile.

In a comparison of the germination of segregating hairy-neck lines, homozygous hairy-neck lines, and homozygous smooth-neck lines, no differences were observed.

In study of the height of plants and of vigor as measured by tillering, it was found that in crosses between the three selections and wheat the smooth-neck segregates were invariably taller than the hairy-neck segregates from the same cross. It was also found that heterozygous hairy-neck segregates were taller than homozygous hairy-neck segregates. In all cases smooth-neck plants from these crosses tillered more than comparable hairy-neck plants.

The F_1 hybrids were reciprocally back-crossed with wheat. In all crosses but one, a larger percentage of hairy-neck plants was produced when the F_1 hybrid was used as a female parent. In the one exception there was practically no difference. There was good agreement among the data secured by back-crossing, the F_2 segregation, and the breeding behavior of the F_3 lines.

A study of differential functioning of pollen grains was made by using mixtures of pollen of Selection C and one of three varieties of wheat. The florets were emasculated and either pollinated with a mixture of pollen or pollinated first with pollen from Selection C and about 2 minutes later with pollen from wheat. The results indicated that the pollen cells of Selection C are less viable or that the pollen tube grows more slowly than in wheat. There was apparently no discrimination on the part of the egg toward either type of gamete.

Since the hairy-neck plants are irregular in their breeding behavior, it seems logical to represent their chromosomal constitution by the expression $2n + 2$ rather than by $2n$, indicating no homologue in wheat for the rye chromosome carrying the hairy-neck factor. It may then be assumed that the hairy-neck plants produce $n + 1$ gametes and that occasionally the rye chromosome is lost, giving a gamete of n consitution. The union of $n + 1$ and n gametes results in a zygote similar to that produced by a cross of a hairy-neck selection × wheat, and the union of n gametes produces a plant which cannot be distinguished from wheat.

THE COMPARATIVE EFFECTIVENESS, IN THE DAIRY RATION, OF SUPPLEMENTS OF PHOSPHORUS IN THE FORM OF ORTHOPHOSPHORIC ACID, MONOSODIUM, DISODIUM, TRISODIUM PHOSPHATES, AND BONE MEAL [1]

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INTRODUCTION

Studies of calcium and phosphorus metabolism carried on for a number of years at the United States Dairy Experiment Station at Beltsville, Md., have shown that a proper adjustment of the mineral content of the ration is of vital importance to health, milk production, and reproduction in the dairy cow.

The work of Shohl (5)[2] with rats a few years ago suggested the possibility that the assimilation of calcium and phosphorus might be affected by a change in the acid-base ratio of the ration. Shohl found the greatest retention of calcium and phosphorus in rats on a neutral diet. The neutral diet was obtained by adding orthophosphoric acid to an alkaline ration. The alkaline ration alone produced the symptoms of tetany and the acid ration (made by adding phosphoric and hydrochloric acid to the alkaline ration) produced the symptoms of rickets.

One of the writers (6) has shown that for favorable assimilation of calcium and phosphorus by dairy cows the calcium-phosphorus ratio should not be too wide. Dairy rations of alfalfa hay and grain often have a rather large proportion of calcium as compared with phosphorus.

The experiments here reported were undertaken (1) to study the effect of variations in the alkalinity of the ration on the calcium and phosphorus metabolism in cows, and (2) to determine the form in which supplements of phosphorus could best be supplied. In the first experiment the ration was supplemented with soluble phosphates; in the second experiment bone meal was used. An attempt was made to eliminate the effect of any organic food constituents by feeding a uniformly good quality of hay and grain throughout.

FIRST EXPERIMENT: ORTHOPHOSPHORIC ACID, MONOSODIUM, DISODIUM, AND TRISODIUM PHOSPHATES AS SUPPLEMENTS OF PHOSPHORUS

In the first experiment a basal ration somewhat low in phosphorus was used and phosphorus supplements were added in the form of orthophosphoric acid, monosodium, disodium, and trisodium phosphates. These were added in such amounts as to maintain a calcium-

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phosphorus ratio in the feed below 1.50, preferably about 1.25. Under such conditions it was hoped that some superiority of one form of supplement over another would be apparent.

Calculated, according to Shohl, on the basis of inorganic acids and bases in the ration, 100 grams of this basal ration was equivalent to 63.3 cubic centimeters normal alkali. The amount of phosphoric acid added was in no case sufficient to neutralize the alkalinity. This method of calculating the reaction of the ration, however, disregards carbonates and organic acids. It may be said to give a rough idea of the titratable alkalinity of the ration, but it gives no idea of what the hydrogen-ion concentration would be in the kind of watery extract that is formed when such a ration is introduced into the alimentary tract. The amounts of orthophosphoric acid and of trisodium phosphate used in this experiment may well have been sufficient to produce definite changes in the hydrogen-ion concentration of the alimentary contents in the early stages of digestion.

The provision for additional phosphorus, at least in the form of the more neutral supplements, has proved valuable and the results have led to certain conclusions which will be discussed later.

EXPERIMENTAL PROCEDURE

For this experiment three Holstein cows were used. Cow 265 was a purebred, and cows A–37 and A–40 were grades. Cows A–37 and A–40 were about 4 years old, and cow 265 was 9 years old. All three cows were pregnant and in the fourth month of lactation when the experiment was started, but cows A–37 and 265 aborted early in the experiment after about 2 months of pregnancy. They were bred again and, at the end of the experiment, cow A–37 had completed 5 months of pregnancy; cow A–40, 7 months; and cow 265, 1.5 months.

The experiment began September 20 and ended March 13, a period of 25 weeks. During the first 4 weeks a basal ration was fed, consisting of U.S. No. 1 grade alfalfa hay and a grain mixture (whole yellow corn meal, 40 parts; wheat bran, 30 parts; soybean meal, 20 parts; linseed meal, 10 parts; and sodium chloride, 1 part). The cows were given as much feed as they would "clean up" and an effort was made to maintain about equal consumption of grain and hay.

Because of the hot weather at this time (early fall) it was difficult to induce the animals to consume sufficient feed to meet their energy requirements, particularly in the case of cow 265. This cow was offered a little timothy hay. She seemed to relish it, and since it had been observed in other experiments at this station (*2*) that animals at times indicated a preference for timothy hay after prolonged periods of alfalfa-hay feeding, it was decided to give all the cows a feeding period on mixed timothy and alfalfa hay. Accordingly, for the next 4 weeks, half of the alfalfa hay of the basal ration was replaced by U.S. No. 1 grade timothy hay. During the following 2 weeks the basal ration was again fed.

During the last 15 weeks of the experiment different phosphorus supplements (equivalent to about 25 to 27 grams of phosphorus daily) were added to the basal ration for periods of 3 weeks each, to learn the effect of varying the alkalinity of the ration.

Beginning with the ninth week, the cows were exercised 10 minutes daily until the end of the experiment. At about that time their appetites began to improve and subsequently their rate of food con-

sumption became steadier. This may be attributed to a combination of factors—exercise, cooler weather, and possibly the feeding of phosphorus supplements.

The weights of the cows at the beginning and end of the experiment were, respectively: Cow A–37, 561 and 651 kilograms; cow A–40, 504 and 577 kilograms; cow 265, 634 and 604 kilograms. The loss of weight by cow 265 was due to the fact that she would not eat sufficient feed to meet her energy requirements.

Hay and grain were fed twice a day and the cows were milked three times a day. Detailed analyses of the feeds are omitted. The alfalfa hay contained approximately 1.5 percent calcium, 0.2 percent phosphorus, and 2.4 percent nitrogen. The timothy hay contained about 0.35 percent calcium, 0.12 percent phosphorus, and 0.82 percent nitrogen. The average grain mixture contained about 0.13 percent calcium, 0.7 percent phosphorus, and 3.4 percent nitrogen. The phosphorus content of the grain was increased by the addition of phosphorus supplements, and was then between 0.9 and 1 percent.

Chemically pure materials were used as supplements and were mixed with the grain. Three parts of sirupy orthophosphoric acid were diluted with 2 parts of water and dropped on the grain as fed each day. During the last 3 weeks of the experiment the phosphoric acid was thoroughly kneaded into the grain mixture to insure actual consumption of the acid.

<div align="center">EXPERIMENTAL RESULTS</div>

Table 1 shows the average weekly feed consumption, milk yield, percentage of calcium and phosphorus in the milk, and the calcium and phosphorus balances for each cow during the different feeding periods. The figures for assimilated calcium and phosphorus were calculated as described in a previous publication (*3*).

Both calcium and phosphorus values indicate that the different feeds affected the composition of the milk, probably through changes in the composition of the blood. The phosphorus content of the milk shows a fairly definite tendency to be a little higher during the periods in which the phosphate supplements were fed than at other times. The calcium in the milk is noticeably higher when the cows were on the basal ration than when grain, alfalfa, and timothy were fed, and also shows a tendency to be higher on the orthophosphoric acid supplement than on trisodium phosphate. In the case of cow A–40 this latter tendency is partly masked by the general tendency for the milk calcium to increase during the latter part of lactation, due perhaps to the decreasing milk yield.

The graphs in figure 1 show the fluctuation in the calcium and phosphorus content of the body which occurred during the course of the experiment. The calcium and phosphorus graphs are drawn on different scales, in the ratio of calcium to phosphorus in bone. A variation of 100 grams of calcium corresponds to a variation of 46 grams of phosphorus. If the calcium and phosphorus balances signify a building up or breaking down of bone material only in the body, then the graphs should follow an identical course. The fact that considerable divergence is shown between the calcium and phosphorus graphs indicates some difference in the storage possibilities of these two elements in the body, or in the intestinal tract.

Table 1.—*Average weekly feed consumption, milk yield, percentage of calcium and phosphorus in the milk, and the calcium and phosphorus balances for the 3 cows during the different feeding periods*

Ration, length of feeding period, and cow no.	Feed consumed Grain	Feed consumed Hay	Milk produced Yield	Composition Calcium	Composition Phosphorus	Calcium In urine and feces	Calcium In milk	Calcium In feed	Calcium Balance	Calcium Assimilation (Grams)	Calcium Assimilation (Pct.)	Phosphorus In urine and feces	Phosphorus In milk	Phosphorus In feed	Phosphorus Balance	Phosphorus Assimilation (Grams)	Phosphorus Assimilation (Pct.)	Calcium-phosphorus ratio in feed
	Kg	*Kg*	*Kg*	*Pct.*	*Pct.*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Pct.*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Pct.*	
Basal ration (4 weeks):																		
Cow A-37	66.5	65.3	143.1	0.121	0.111	874.4	172.8	1,063.7	+16.5	189.3	17.8	433.2	150.2	586.3	−6.1	153.1	26.1	1.81
Cow A-40	60.8	51.0	114.5	.125	.107	742.3	142.3	843.2	−41.9	100.9	12.0	433.0	122.5	520.2	−35.3	87.2	16.8	1.62
Cow 265	66.6	50.4	172.5	.104	.098	700.7	179.6	822.4	−57.9	121.7	14.8	429.0	168.0	549.7	−45.3	120.7	21.9	1.50
Alfalfa, timothy, and grain (4 weeks):																		
Cow A-37	70.0	70.0	135.7	.115	.116	595.5	156.7	727.0	−25.2	131.5	18.1	452.4	157.3	575.5	−34.2	123.2	21.4	1.26
Cow A-40	63.0	55.0	122.1	.123	.109	504.2	150.8	587.2	−67.8	83.0	14.1	417.7	133.4	505.5	−45.6	87.8	17.4	1.16
Cow 265	70.0	47.3	151.1	.099	.094	469.5	149.6	533.1	−86.0	63.6	11.9	468.5	142.8	540.0	−71.3	71.4	13.2	.99
Basal ration (2 weeks):																		
Cow A-37	70.0	70.0	130.7	.119	.115	966.3	156.2	1,127.5	+5.0	161.2	14.3	472.7	151.0	626.0	+2.3	153.3	24.5	1.80
Cow A-40	63.0	54.0	117.3	.127	.106	756.3	148.9	880.8	−24.4	124.5	14.1	428.8	124.9	548.4	−5.3	119.6	21.8	1.61
Cow 265	70.0	42.8	143.4	.101	.092	594.2	144.8	697.9	−41.1	103.7	14.9	451.1	132.7	560.8	−23.0	109.8	19.6	1.24
Basal ration, plus orthophosphoric acid (3 weeks):																		
Cow A-37	70.0	63.0	126.2	.120	.118	894.3	151.8	1,018.2	−27.9	123.9	12.2	662.8	148.8	795.3	−16.3	132.6	16.7	1.28
Cow A-40	63.0	56.0	115.9	.126	.110	767.1	145.6	905.6	−7.1	138.5	15.3	612.0	127.1	733.7	−5.4	121.7	16.6	1.23
Cow 265	70.0	49.5	153.9	.105	.100	685.0	161.5	818.9	−27.6	133.9	16.4	641.4	153.7	769.8	−25.3	128.4	16.7	1.06
Basal ration, plus monosodium phosphate (3 weeks):																		
Cow A-37	70.0	63.0	115.4	.118	.118	872.2	136.2	1,016.1	+7.7	143.9	14.2	640.8	135.8	799.7	+23.1	159.0	19.9	1.27
Cow A-40	63.0	56.0	110.2	.136	.110	728.0	150.2	904.2	+26.0	176.2	19.5	580.8	121.6	718.5	+16.1	137.6	19.2	1.29
Cow 265	70.0	63.0	173.5	.106	.099	808.2	183.3	1,016.1	+24.6	207.9	20.5	617.4	171.1	799.7	+11.2	182.3	22.8	1.27
Basal ration, plus disodium phosphate (3 weeks):																		
Cow A-37	61.7	63.0	97.5	.119	.116	861.2	115.6	1,011.6	+34.8	150.4	14.9	566.9	112.7	687.8	+8.2	120.9	17.6	1.47
Cow A-40	60.0	56.0	102.8	.139	.111	730.9	143.3	905.4	+31.2	174.5	19.3	530.3	114.5	658.1	+13.3	127.8	19.4	1.38
Cow 265	68.3	61.5	166.6	.106	.097	779.4	176.4	975.7	+19.9	196.4	20.1	575.5	161.1	735.9	−.7	160.4	21.8	1.33
Basal ration, plus trisodium phosphate (3 weeks):																		
Cow A-37	61.5	57.3	94.4	.114	.115	799.7	107.6	932.7	+25.4	132.9	14.3	561.5	108.0	657.8	−12.5	96.4	14.6	1.42
Cow A-40	53.3	50.3	85.9	.142	.109	643.7	124.7	818.1	+49.7	174.4	21.3	472.8	96.1	571.8	+2.9	99.1	17.3	1.43
Cow 265	70.0	63.0	170.3	.103	.097	848.8	176.0	1,027.7	+2.9	178.9	17.4	586.7	164.6	744.6	−6.7	157.9	21.2	1.38
Basal ration, plus orthophosphoric acid (3 weeks):																		
Cow A-37	63.0	56.0	83.0	.120	.117	773.5	99.3	912.0	+39.2	138.5	15.2	651.2	97.1	718.0	−30.3	66.9	9.3	1.27
Cow A-40	53.3	46.6	65.2	.154	.110	609.4	100.2	761.4	+51.8	152.0	20.0	551.0	71.7	624.1	+1.4	73.1	11.7	1.22
Cow 265	68.3	63.0	160.4	.104	.094	868.5	166.9	1,022.7	−12.7	154.2	15.1	655.9	150.9	764.0	−42.8	109.1	14.1	1.34

While this experiment was undertaken for the purpose of investigating the effect of variations in the alkalinity of the ration on the calcium and phosphorus metabolism of cows, yet, as the experiment progressed, other factors were indicated as of equal, or possibly greater, importance than the reaction of the ration, namely, the quantity and proportion of calcium and phosphorus in the ration.

A basal ration of alfalfa hay and grain probably does not contain the optimum proportion of phosphorus to calcium for high milk production, even though the grain (containing 30 percent wheat bran) has a fairly high phosphorus content. This is indicated by the fact that all the cows showed excessive losses of phosphorus from the body while on this ration during the first period. Insufficient phosphorus intake and rather generous milk flow are probably the factors causing the negative balances at this time. The calcium-phosphorus ratio in this period ranged from 1.5 to 1.8.

In the second period the intake of calcium was greatly reduced by the substitution of timothy hay for half of the alfalfa in the basal ration; while, in the third period, the calcium intake was increased by a return to the basal ration. The calcium and phosphorus balances became more negative in the second period, and less negative in the third period, but it is doubtful whether these changes in the balances have any physiological significance. The matter can be more profitably discussed in connection with some of the results of the second experiment, which will be given later.

With the introduction of the phosphorus supplements during the remainder of the experiment the phosphorus intake was materially increased. This increased phosphorus intake did not appear to be very effective in preventing mineral losses, however, when supplied in the form of orthophosphoric acid, as in the fourth and eighth periods. The quantities of
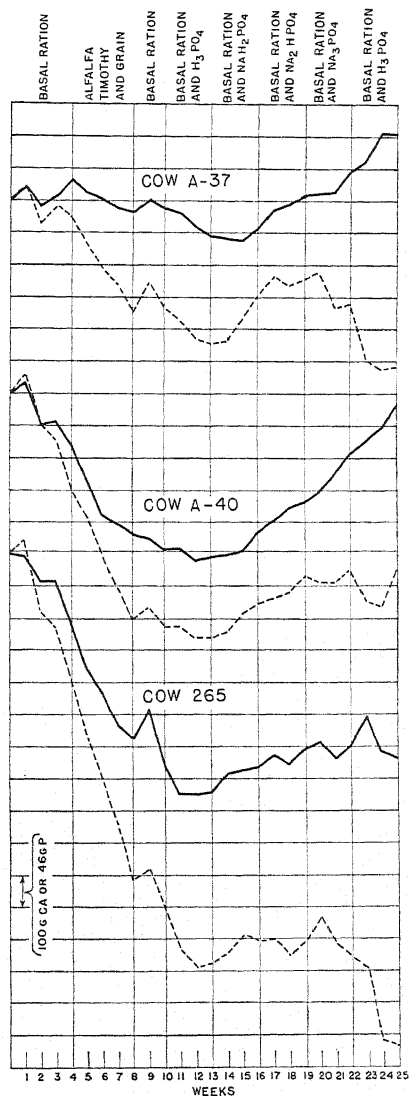

FIGURE 1.—Fluctuation in the content of calcium and phosphorus in the bodies of the cows during the course of the first experiment. The solid line represents calcium; the dotted line, phosphorus. The divisions on the ordinate correspond to 100 grams of calcium or 46 grams of phosphorus, the ratio in which the elements are present in bone.

phosphorus in the ration and the calcium-phosphorus ratio assumed more appropriate values (average 1.19). In spite of this the animals continued to lose calcium and phosphorus in the fourth period. In the eighth period, when the same supplement was fed, the phosphorus losses were considerable in the case of cows A–37 and 265. Hart (*1*) recently observed, after administering hydrochloric acid to cows, that there was a divergence of the calcium excretion from the feces to the urine but no improvement in calcium balances.

During the fifth and sixth periods, monosodium and disodium phosphates were used as supplements and the effect was marked. Not only were calcium and phosphorus losses checked in the case of all animals but a distinct recovery of mineral stores was initiated which extended even into the seventh period. This occurred without any great reduction in milk yield and while the calcium-phosphorus ratio was being maintained at the above-mentioned appropriate value.

The effect of trisodium phosphate as a supplement was somewhat irregular. Possibly the alkalinity of this material was unfavorable for mineral absorption.

These results would seem to indicate that by merely supplying a suitable neutral phosphorus supplement in sufficient quantity and in proper ratio to the calcium present in the ration (that calcium being already present in an available form and in generous amount), a phosphorus-deficient ration can be made adequate and equilibrium or positive balances can be obtained. It seems reasonable to assume, since the mature high-producing cow's chief need for calcium and phosphorus is to secrete them in the milk, that the ration which she receives should contain calcium and phosphorus in approximately the same proportion that they occur in milk, namely, about 1.1:1. If calcium and phosphorus are supplied in assimilable form and in sufficient quantity and in the proportion present in milk (the ration being satisfactory in other known respects), then, the writers believe, much will have been done to improve the mineral nutrition of the cow.

Phosphorus, in the form of disodium phosphate, was fed to dry cows at this station several years ago (*4*). Very definite increases in milk yield during the subsequent lactations were noted. At that time, however, only a few short-time balances were followed where the cows were receiving a mineral phosphorus supplement.

Since the supplements monosodium and disodium phosphate have brought about so marked a retention of calcium and phosphorus in animals that have suffered considerable mineral losses, they should also be effective in preventing such losses in animals in a better state of nutrition. That such losses, however, are not entirely preventable is evident from an experiment conducted at this station recently (*7*). In this instance two cows giving 21 to 28 kilograms of milk daily were fed the best ration that the writers could devise, including a supplement of disodium phosphate. With average daily calcium and phosphorus intakes of 129 and 117 grams, respectively, these cows were slowly but steadily losing calcium and phosphorus from their bodies. It seems impossible to escape the conclusion recently stated by Hart (*1*), that "in the early period of lactation, *especially with high milk flow*, the calcium assimilation from the digestive tract is insufficient to meet the needs of mammary secretion and

the skeleton is drawn upon, with a negative calcium balance result-
ing." The interdependence of calcium and phosphorus metabolism
involves a simultaneously lowered phosphorus assimilation.

SECOND EXPERIMENT: BONE MEAL AS A PHOSPHORUS SUPPLEMENT

A second experiment, conducted in a somewhat different manner,
was completed about a year later. In this experiment bone meal
was used as a supplement instead of the soluble phosphates used in
the first experiment. Bone meal is so often employed as a source of
calcium and phosphorus for cattle that it was thought advisable to
study its effects on the metabolism of these elements; but, as will
appear later, its use introduces experimental complications, which
make it necessary to exercise great caution in drawing conclusions as
to the physiological significance of the results.

EXPERIMENTAL PROCEDURE

Three grade Holstein cows were used in this experiment—cows
A–37, A–43, and A–46. Cows A–37 and A–46 were not pregnant;
cow A–43 had been pregnant for about a month at the end of the
experiment. The animals were from $3\frac{1}{2}$ to 5 years of age.

The experiment began November 5 and ended January 13, a period
of 10 weeks. During the first 3 weeks of the experiment the same
basal ration was used as in the first experiment. During the next 7
weeks supplements of bone meal and disodium phosphate were added
to the basal ration, bone meal the first 3 weeks and phosphate the last
4 weeks. The amounts used were so regulated as to introduce a uni-
form increase in the phosphorus intake, that is, one which, when intro-
duced in the form of sodium phosphate, would keep the calcium-
phosphorus ratio in the feed between 1.1 and 1.5. Obviously when
bone meal was used it was impossible to make this correction in the
calcium-phosphorus ratio of the feed. These supplements were added
to the grain mixture, and in the case of bone meal, represented about
3.8 percent, and in the case of the disodium phosphate, about 6.5 per-
cent of the mixture.

The weights of the cows at the beginning and end of the experiment
were respectively: Cow A–37, 568 and 575 kilograms; cow A–43, 439
and 469 kilograms; cow A–46, 452 and 461 kilograms.

Hay and grain were fed twice a day and the cows were milked
three times a day.

The alfalfa hay contained about 1.5 percent calcium and 0.2 per-
cent phosphorus, the grain 0.13 percent calcium and 0.7 percent
phosphorus. Chemically pure disodium phosphate and a high grade
of bone meal were used to supplement the grain mixture and were
thoroughly mixed with it. The bone meal contained 29.45 percent
calcium and 14.06 percent phosphorus.

EXPERIMENTAL RESULTS

The milks secreted showed a uniform slight increase in phosphorus
content when bone meal was fed (table 2).

TABLE 2.—*Weekly feed consumption, percentage of calcium and phosphorus in the milk, and the calcium and phosphorus balances for the 3 cows during the different feeding periods*

COW A-37

Ration and period	Feed consumed		Milk produced			Calcium						Phosphorus						Calcium-phosphorus ratio in feed
	Grain	Hay	Yield	Composition		In urine and feces	In milk	In feed	Balance	Assimilation		In urine and feces	In milk	In feed	Balance	Assimilation		
				Calcium	Phosphorus					Grams	Pct.					Grams	Pct.	
	Kg	*Kg*	*Kg*	*Pct.*	*Pct.*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*		*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Pct.*	
Basal ration:																		
First week	77	77	155.9	0.124	0.119	1,052.3	193.3	1,349.7	+104.1	297.7	22.0	479.7	185.5	679.6	+14.4	199.9	29.4	2.0
Second week	77	77	153.0	.124	.116	1,050.0	189.7	1,352.8	+113.1	302.8	22.4	504.8	177.5	697.9	+15.6	193.1	27.7	2.0
Third week	77	77	149.4	.129	.118	1,130.3	192.7	1,351.9	+28.9	221.6	16.4	542.7	176.3	708.6	−10.4	165.9	23.4	2.0
Average	77	77	152.8	.126	.118	1,077.5	191.9	1,351.5	+82.0	273.9	20.3	509.1	179.8	695.4	+6.5	186.3	26.8	2.0
Basal ration, plus bone meal:																		
Fourth week	77	77	149.5	.120	.121	1,884.1	179.4	2,184.1	+120.0	300.0	13.7	844.6	180.9	1,007.1	+71.6	252.5	23.0	2.0
Fifth week	77	77	142.9	.124	.120	1,880.2	177.2	2,158.1	+100.7	277.9	12.9	857.0	171.5	1,064.0	+35.5	207.0	19.5	2.0
Sixth week	60.5	66	132.4	.119	.119	1,529.7	157.6	1,722.6	+35.3	192.9	11.2	711.5	157.6	845.7	−23.4	134.2	15.9	2.0
Average	71.5	73.3	141.6	.121	.120	1,764.7	171.4	2,021.6	+85.5	256.9	12.6	804.4	170.0	1,002.3	+27.9	197.9	19.5	2.0
Basal ration, plus sodium phosphate:																		
Seventh week	66	77	139.1	.117	.118	1,203.4	162.7	1,262.1	−104.1	58.7	4.7	810.8	164.1	949.4	−31.5	132.6	14.0	1.3
Eighth week	60.5	77	130.6	.117	.114	1,117.9	152.8	1,258.6	−12.1	140.7	11.2	715.4	148.9	808.5	+4.2	153.1	17.6	1.4
Ninth week	55	77	130.3	.119	.118	1,152.5	155.1	1,248.9	−58.7	95.4	7.7	750.8	153.8	838.5	−66.1	87.7	10.5	1.5
Tenth week	66	77	134.7	.119	.117	1,021.2	160.3	1,231.0	+49.5	209.8	17.0	794.3	157.6	944.7	−7.2	150.4	15.9	1.3
Average	61.9	77	133.7	.118	.117	1,123.8	157.7	1,250.2	−31.3	126.4	10.2	769.3	156.1	900.3	−25.2	131.0	14.5	1.4

COW A-43

Ration and period	Feed consumed		Milk produced			Calcium						Phosphorus						Calcium-phosphorus ratio in feed
Basal ration:																		
First week	70	63	149.0	0.120	0.095	878.4	178.8	1,113.2	+56.0	234.8	21.1	457.4	141.6	603.5	+4.5	146.1	24.2	1.8
Second week	70	63.5	148.6	.121	.091	869.2	179.8	1,116.0	+67.0	246.8	22.1	463.0	135.2	610.1	+11.9	147.1	24.1	1.8
Third week	70	49.5	139.5	.122	.096	852.1	170.2	895.8	−126.5	43.7	4.9	501.5	133.9	602.2	−33.2	100.7	16.7	1.5
Average	70	58.5	145.7	.121	.094	866.6	176.3	1,041.7	−1.2	175.1	16.0	474.0	136.9	605.3	−5.6	131.3	21.7	1.7

Basal ration, plus bone meal:																		
Fourth week	70	58.5	145.5	.114	.098	1,539.4	165.9	1,823.6	+118.3	294.2	15.6	769.7	142.6	981.8	+69.5	212.1	21.6	1.9
Fifth week	70	63	150.1	.120	.098	1,625.7	180.1	1,854.7	+48.9	229.0	12.3	781.5	147.1	953.1	+24.5	171.6	18.0	1.9
Sixth week	70	63	143.6	.121	.100	1,588.5	173.8	1,787.1	+25.0	198.8	11.1	819.6	143.6	951.7	-11.5	132.1	13.9	1.9
Average	70	61.5	146.4	.118	.099	1,584.5	173.3	1,821.8	+64.1	237.3	13.0	790.3	144.4	962.2	+27.5	171.9	17.8	1.9
Basal ration, plus sodium phosphate:																		
Seventh week	70	63	141.5	.114	.100	940.2	161.3	1,052.1	-48.8	112.5	10.7	868.2	141.5	969.4	-40.3	101.2	10.4	1.1
Eighth week	70	63	146.1	.115	.099	927.9	168.0	1,056.0	-39.9	128.1	12.1	819.5	144.6	955.6	-8.5	136.1	14.2	1.1
Ninth week	70	63	145.3	.117	.098	895.6	170.0	1,052.8	-12.8	157.2	14.9	543.4	142.4	997.9	+12.1	154.5	15.5	1.1
Tenth week	70	63	149.2	.114	.100	907.5	170.1	1,052.8	-50.8	119.3	11.6	833.3	149.2	961.7	-17.8	131.4	13.6	1.1
Average	70	63	145.5	.115	.099	917.8	167.4	1,047.1	-38.1	129.3	12.3	841.1	144.4	971.9	-13.6	130.8	13.4	1.1

COW A-46

Basal ration:																		
First week	70	70	130.6	0.127	0.113	1,002.5	165.9	1,227.3	+58.6	224.5	18.3	477.2	147.6	617.8	-7.0	140.6	22.8	2.0
Second week	70	70	129.9	.124	.109	980.4	161.1	1,229.8	+88.3	249.4	20.3	479.5	141.6	634.4	+13.3	154.9	24.4	0.9
Third week	60	60	120.6	.126	.107	914.4	152.0	1,066.5	+.1	152.1	14.3	499.1	129.0	623.7	-4.4	124.6	20.0	1.7
Average	70	66.6	127.0	.126	.110	965.8	159.7	1,174.4	+49.0	208.7	17.6	485.3	139.4	625.3	+.6	140.0	22.4	1.9
Basal ration, plus bone meal:																		
Fourth week	70	60	124.4	.119	.115	1,589.6	148.0	1,848.6	+110.9	258.9	14.0	794.8	143.1	984.9	+47.0	190.1	19.3	1.9
Fifth week	70	70	126.4	.124	.113	1,715.4	156.7	1,961.9	+89.8	246.5	12.6	796.3	142.8	967.2	+28.1	170.9	17.7	2.0
Sixth week	70	70	122.1	.125	.115	1,721.3	152.6	1,894.3	+20.4	173.0	9.1	538.1	140.4	965.8	+12.7	127.7	13.2	2.0
Average	70	66.6	124.3	.123	.114	1,675.4	152.4	1,961.6	+73.7	226.1	11.9	809.7	142.1	972.6	+20.8	162.9	16.7	2.0
Basal ration, plus sodium phosphate:																		
Seventh week	65	70	117.6	.123	.116	1,071.1	144.6	1,153.6	-62.1	82.5	7.2	801.1	136.4	923.3	-14.2	122.2	13.2	1.2
Eighth week	65	70	116.7	.121	.114	1,016.5	141.2	1,157.0	-.7	140.5	12.1	814.0	133.0	909.1	-37.9	95.1	10.5	1.3
Ninth week	65	70	106.4	.122	.109	1,031.8	129.8	1,154.0	-7.6	122.2	10.6	776.1	116.0	948.1	+56.0	172.0	18.1	1.2
Tenth week	60	70	113.7	.120	.113	1,001.1	136.4	1,119.2	-18.3	118.1	10.6	798.2	128.5	855.2	-71.5	57.0	6.7	1.3
Average	63.7	70	113.6	.122	.113	1,030.1	138.0	1,146.0	-22.2	115.8	10.1	797.4	128.5	908.9	-16.9	111.6	12.1	1.3

Figure 2 shows the fluctuation in calcium and phosphorus content in the bodies of the cows during the course of the experiment.

From a study of table 2 and figure 2 it is evident that the cows retained calcium during the period in which they were on the basal ration (except cow A–43) and that they retained even more calcium as well as phosphorus during the bone-meal supplement period but lost both calcium and phosphorus during the sodium-phosphate supplement period.

The effects of changes in rations on calcium and phosphorus balances noted here are typical of those observed in all balance experiments where the intakes of calcium and phosphorus are varied markedly during the experiment. It is probable, however, that these changes do not represent alterations in the amounts of calcium and phosphorus stored in the bones or other internal tissues of the animal, but changes primarily in the amounts of calcium and phosphorus held in mechanical suspension in the fluid contents of the stomachs and intestines.

The bovine intestinal tract has a very complicated form, consisting of the four stomachs, one of which has highly corrugated walls, in addition to the large intestine and the very long and convoluted small intestine. There can be no doubt that considerable proportions of such insoluble material as tricalcium phosphate, or of any calcium and phosphorus compounds capable of forming tricalcium phosphate, when introduced into this tract with the food, are likely to remain in it for long periods. Furthermore, the normal amount of the bovine intestinal contents or "fill" is very large. In the case of three cows of the Jersey type recently slaughtered at this station after they had fasted for 24 hours, the average amount of fill was 52 kilograms. It is probable that in the case of Holstein cows on full feed the fill would be about 100 kilograms.

FIGURE 2.—Fluctuation in the content of calcium and phosphorus in the bodies of the cows during the course of the second experiment. The solid line represents calcium; the dotted line, phosphorus. The divisions on the ordinate correspond to 100 grams of calcium or 46 grams of phosphorus, the ratio in which these elements are present in bone.

Some idea as to how much calcium is likely to be retained in the intestinal contents and how long this element is likely to go on increasing there during a period of bone-meal feeding may be obtained from the figures given for the calcium in the urine and feces of cows A–43 and A–46 in table 2. Cow A–37 is omitted from this discussion because she was badly off feed in the last of the periods in which bone meal was fed. It will be seen that the calcium content of the urine and feces of cows A–43 and A–46 did not reach its height until the second or third week of bone-meal feeding.

Unfortunately the percentage of calcium in the feces alone was not determined. However, the percentage of calcium in the amount of feces and urine discharged weekly and mixed with a small amount of wash water was determined; and, as the urine contains very little calcium as compared with the feces, these figures give a rough idea of

the amounts of calcium contained in the intestinal contents at the beginning and end of the bone-meal feeding period. The actual amount of calcium contained in the fill at the beginning of this period, and the actual increase during its progress would, of course, be considerably larger than these figures indicate.

The average amount of calcium in the urine and feces of cows A–43 and A–46 during the last period that they were on the basal ration was 0.238 percent, and during the last period on bone meal it was 0.378 percent. On the supposition that the fill amounted in each case to 100 kilograms, this would mean that the intestinal contents of the cows had 238 grams of calcium at the beginning of the bone-meal feeding period and 378 grams at the end, and that the calcium retained in the intestinal tract increased by 140 grams during this period. This increase accounts for most of the increase in body calcium as shown by the balance results. It is not unreasonable to suppose, therefore, that the positive calcium and phosphorus balances in the second period of the experiment represent merely an accumulation of these elements in the intestinal contents; and that the negative balances in the third period represent a gradual loss of this accumulation after the daily intake had been decreased.

It is not unlikely that a somewhat similar explanation may account for the changes in the balances which took place in the first, second, and third periods of the first experiment where the intake of calcium was reduced by the feeding of timothy hay. This interpretation would not appear to invalidate the conclusions expressed in respect to the different phosphate supplement periods where the intakes of calcium and phosphorus were maintained quite uniformly constant.

SUMMARY AND CONCLUSION

Two experiments made to determine the relative value of certain soluble phosphates and bone meal as phosphorus supplements in dairy rations, are reported. In the first experiment the phosphorus supplements were orthophosphoric acid, monosodium, disodium, and trisodium phosphates; in the second experiment bone meal was used. During the first period of each experiment the same basal ration was fed, but the first experiment was started in late September, when hot weather and annoyance from flies reduced the consumption of feed, whereas the second experiment was started in the cooler weather of November. The result was smaller intakes of calcium and phosphorus and negative balances in the first experiment and larger intakes of calcium and phosphorus and positive balances in the second experiment.

Dairy rations of alfalfa hay and grain often have a rather large proportion of calcium as compared with phosphorus. The experiments here reported indicated that calcium and phosphorus balances of cows fed on such rations may sometimes be rendered more positive by adding orthophosphates to them. This improvement is more marked when the nearly neutral phosphates disodium phosphate, and monosodium phosphate are used than when orthophosphoric acid or trisodium phosphate is used.

Large increases in the amount of calcium and phosphorus received by cows in their rations are likely to be followed by more positive calcium and phosphorus balances; decreases, by less positive balances.

There is reason to believe, however, that considerable quantities of calcium and phosphorus may be retained as insoluble tricalcium phosphate for several weeks in the intestinal tracts of cows, and there is no way of knowing what proportion of positive calcium and phosphorus balances is to be explained in this way and what proportion really represents a gain in bone tissue. Changes in the balances, following large changes in the calcium and phosphorus intake, and lasting not more than a few weeks, should not therefore be taken as any certain indication of changes in the assimilation of calcium and phosphorus from the intestinal tract.

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INHERITANCE OF RESISTANCE TO LOOSE SMUT IN CERTAIN WHEAT CROSSES [1]

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INTRODUCTION

During recent years the principles of Mendelism have been applied extensively in the production of new types of plants possessing resistance to various diseases. The results of this mode of attacking the disease problem have been highly favorable. Old varieties are gradually giving way to newer types equal to or exceeding the old in quality and productivity as well as possessing resistance to one or more diseases.

Loose smut in wheat, *Ustilago tritici* (Pers.) Jens., while not as serious a problem in Utah as the covered smut (*Tilletia* spp.) has,
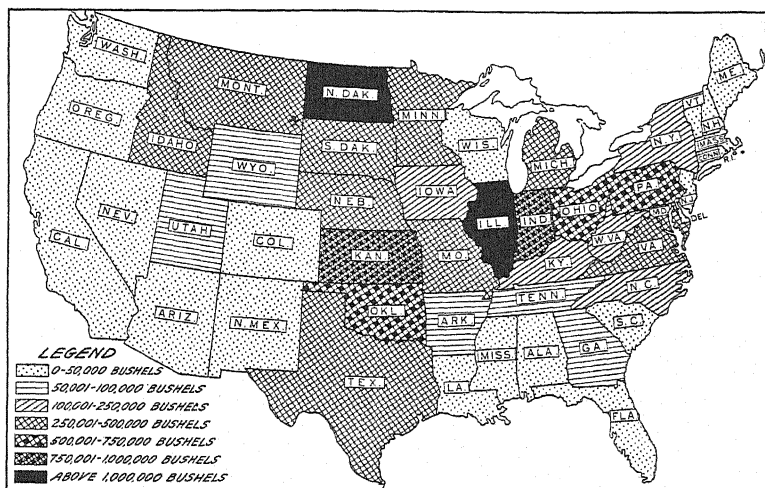
FIGURE 1.—Estimated average annual loss of wheat due to loose smut, by States, 1917–26, inclusive. From Journal of Agricultural Research 39: 314 (1929).

according to Tapke (*16*) [3] caused an average annual loss in this State of between 50,000 and 100,000 bushels of wheat (fig. 1). The various methods advocated for the control of loose smut in wheat, with the exception of the use of resistant varieties and hot-water treatments, have been either impractical of application or ineffective in control, or both (*16*).

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According to Tapke (*16*) the modified hot-water treatment devised by Freeman and Johnson (*4*) has generally been the method recommended. While this method is effective, if properly carried out, it is rather complicated and tedious to apply, especially for farmers, who usually are not properly equipped. Because of this and the fact that the disease frequently escapes observation, seed treatment for the control of loose smut is seldom practiced; as a result, the disease is allowed to go unchecked. The development of a resistant variety possessing the other desirable characteristics of locally grown spring wheats would be a decided advantage to the farmers in combating the disease.

REVIEW OF LITERATURE

A number of studies have been made on the comparative resistance of wheat varieties to loose smut. Tapke (*16*) gives an account of his studies on varietal resistance to this disease. It is evident from his work, as well as that of others, that some varieties exhibit greater resistance than others. Matsuura (*12*) reviews a report, Washington Agricultural Experiment Station Bulletin 155, presumably on the inheritance of resistance to *Ustilago tritici*. However, the original article refers only to smut and does not state whether it was *U. tritici* or *Tilletia tritici*. An article by T. Kilduff (*9*) came to the attention of the writers as this manuscript was ready for publication. The writer was unable to give a genetic analysis of inheritance to loose-smut resistance.

EXPERIMENTAL MATERIAL AND METHODS

PARENTAL MATERIAL USED

Varieties and strains of wheat used in these studies were Hope C.I. 8178; Preston C.I. 3081; 01–24, C.I. 11542; Dicklow No. 3; and Federation. These are all classed as *Triticum vulgare* Vill. wheats. The two leading spring varieties grown in Utah are Federation and Dicklow. Dicklow No. 3 is a Utah selection out of the Dicklow variety. This strain is the one used as a check in the wheat-nursery tests conducted at the station. It is more uniform, is less subject to lodging, and is a slightly higher yielder than the ordinary Dicklow variety. Strain 01–24 is a new production from the Utah station. It is a strong-strawed, high-yielding, white-kerneled spring wheat. The parents of this strain are not definitely known. However, it is probably a segregate out of either a Dicklow × Federation or a Dicklow × C.I. 4722 cross. The reaction of this strain to loose smut places some doubt on the possibility of its being out of the Dicklow × Federation cross, as Federation appears to possess no factors for resistance and 01–24 is more resistant than the Dicklow No. 3 strain. There is, however, the possibility that the Dicklow or Federation used in the cross from which 01–24 might have been selected was more resistant than the Dicklow No. 3 or the Federation used in these studies. Selections of Dicklow and Federation made at the Utah station show definitely that these two varieties do possess individuals differing in physiological characters; similar or even greater differences are possible in regard to their reaction to loose smut. A history of the origin and development of Hope C.I. 8178 is given by McFadden (*10*); Preston C.I. 3081 and Federation are described by Clark et al.

(1). The varieties, with contrasted characters studied, are shown in table 1.

TABLE 1.—*Contrasted characters of the parents studied in the crosses*

| Parental variety | Morphological characters | | | Average percentage of infection with loose smut |
	Presence or absence of awns	Chaff color	Grain color	
Hope	Fully awned	White	Red	0
Preston	do	do	do	1.6±0.39
01–24	Short apical awns	Bronze	White	18.55±1.21
Dicklow No. 3	do	White	do	38.47±1.27
Federation	Short beaks	Bronze	do	73.50±1.57

Inoculation experiments conducted in 1928 showed Hope wheat to be completely immune to the inoculum used. Later McFadden *(10)*, who is responsible for the development of this variety, was led to conclude from his observations and tests that Hope wheat is highly resistant, if not immune, to the loose smut occurring on Kota. Other strains and varieties were found to possess varying degrees of resistance. However, Dicklow and Federation were found to be susceptible to the inoculum used. Federation was highly susceptible, whereas Dicklow possessed only a fair degree of resistance. This was somewhat surprising as Dicklow has consistently smutted under natural conditions more than Federation. However, it is undoubtedly due to a type of morphological resistance possessed by the Federation and not by the Dicklow variety. This resistance appears to be of such a nature as to prevent the smut spore from coming in contact with the stigma, such as having closer flowering glumes or having stigmas which are less likely to protrude outside the flowering glume during the blooming period.

INOCULUM USED

One of the difficulties in attempting to place the inheritance of disease resistance on a definite factorial basis is the possibility that the inoculum used may not be of a single physiologic form.

Rodenheiser *(14)* concluded from culture studies on nutrient media that there were physiologic forms of *Ustilago tritici* and *U. nuda* (Jens.) K. and S. In fact, he is of the opinion that *U. tritici* and *U. nuda* are physiologic forms rather than separate species. Whether these or other forms will be different pathogenically remains to be determined. However, there is reason to believe that there are different physiologic forms of *U. tritici*. Humphrey and Tapke *(8)* conclude from cross-inoculation experiments that wheat and rye smuts were identical *U. tritici*. Reed *(13)* reports physiologic strains within *U. avenae* (Pers.) Jens. Faris *(2)* has reported similar results on *U. hordei* (Pers.) K. and S. Tisdale and Johnson *(17)* and Stakman and Christensen *(15)* have demonstrated the existence of physiologic strains of *U. zeae* (Beckm.) Ung. The inoculum used was originally taken from the Dicklow variety, and it apparently spread to Federation and Sevier. Dicklow has been grown at the Utah station for years, and the other varieties, except Federation and Sevier, have shown little or no infection. Some preliminary data secured seem to indicate that the inoculum used in these studies was

comparatively uniform pathogenically. In figure 2 are shown two spikes of wheat, the one healthy and the other infected with loose smut.

EXPERIMENTAL METHODS

Pure-line crosses between Hope × Federation, Hope × Dicklow No. 3, and Preston × 01–24 were made at the Central Experimental Farm (North Logan) in 1928. Pollen from anthers of a single spike was used to pollinate the stigmas of a single spike. The progeny of a single F_1 plant were seeded in the spring of 1929. The kernels were spaced about 2.5 inches apart in a row. Inoculations were made by the time the spikes reached the full bloom stage. In preparing the heads for inoculation, the center and the basal and terminal florets of each spikelet were removed; if awns were present, they were clipped off. The glumes of the remaining florets were then spread apart with small hand forceps, and the stigma was thoroughly dusted with the inoculum. Seedlings were made from each F_2 plant. The number of F_2 plants represented in F_3 rows are shown in the various goodness-of-fit tables. The F_3 rows were sown in randomized blocks with duplicate plantings of the Hope × Federation cross and three replicates of the Hope × Dicklow No. 3 and Preston × 01–24 crosses. A number of parental rows were sown at random over the experimental area. The genetic characters studied were resistant to loose smut, awns, glume, and kernel color. Smut-infection data were based on plant count and not on head count.

FIGURE 2.—*A*, Healthy spike of wheat; *B*, smutted spike. (From Journal of Agricultural Research, 39: 316 (1929).

In studying the goodness of fit, the χ^2 test, as given by Fisher *(3)*, was used.

EXPERIMENTAL RESULTS

PRELIMINARY EXPERIMENTS ON INOCULATION TECHNIC

Some preliminary studies were made in 1928 in order to determine the proper time and method of inoculation to insure maximum infection.

METHODS OF INOCULATION

Chlamydospores were suspended in water and then placed on the stigmas of some of the plants; the powdered inoculum was placed on

the stigmas of others by opening the glumes and applying the spores with a pair of forceps. The third method tried was that of heavily dusting the dry spores on the spikes of plants; and the fourth method was that of dipping the spike in a beaker of water heavily laden with spores of loose smut. Table 2 gives the results of this test.

TABLE 2.—*Percentage of infection obtained from various methods of inoculation (given to the nearest whole percent)* [a]

Method of inoculating	Federation		Dicklow No. 3	
	Total plants	Plants infected	Total plants	Plants infected
	Number	*Percent*	*Number*	*Percent*
Spores placed on stigma:				
Dusted on	73	60	54	52
Suspended in water	21	50	11	57
Spores placed on the spikes:				
Dusted on	59	15	72	34
Suspended in water	22	5	12	0
Check (no inoculation)	17	0	15	0

[a] Data in this table show little difference in the percentage of infection in Dicklow No. 3 and in Federation. The percentage of infection is somewhat high for one and correspondingly low for the other as compared with data in table 1. This is probably due to the fact that the data here are based on single 6-foot rows subject to rather wide variations, whereas data in table 1 represent an average for a number of rows.

It is evident from table 2 that the most effective methods of inoculating were those which involved the placing of the spores directly on the stigmas. However, there was no difference between placing the spores on the stigmas when dry or suspended in water. Dusting the spikes with the spores appeared more effective than dipping them in water containing spores. It will be seen from table 3 (compare with table 4), however, that response to dusting spikes with the inoculum varied with the variety and, therefore, could not be relied upon to give satisfactory results in genetic studies.

TABLE 3.—*Reaction of the wheat varieties and strains to loose smut when the inoculum was dusted on the spikes*

Wheat variety or strain	Total plants	Plants infected	Infection in check plants [a]	Wheat variety or strain	Total plants	Plants infected	Infection in check plants [a]
	Number	*Percent*	*Percent*		*Number*	*Percent*	*Percent*
Dicklow	72	34.7	0.0	Q-250	105	1.9	2.4
Dicklow No. 3	53	34.0	6.1	R-18-5	77	10.4	.0
Dicklow No. 16	61	41.0	.0	R-48-22	82	2.4	.0
Federation	59	15.2	.0	R-S 4-5	106	7.4	.0
Hard Federation	60	.0	.0	R-S 17	72	4.1	.0
Marquis	54	3.6	.0	A-4	70	.0	.0
Alcalde	58	.0	.0	14-85	81	1.2	.0
Onas	52	13.0	.0	G-40	60	.0	.0
Sevier A	37	27.0	.0	G-43-11	75	4.0	.0
Sevier 59	36	16.7	.0	G-48	62	6.4	.0
Sevier 125	31	6.5	.0	G-149	65	3.1	.0
No. 139-3	59	11.9	.0	4-287	67	3.0	.0
No. 146	57	7.0	1.2	5-185	68	7.3	1.0
No. 49-10	43	4.7	.0	9-7	48	10.5	.0
No. 1-174-2	68	10.3	1.6	11-12	80	13.7	.0
01-24	47	10.6	.0	11-88	74	1.5	.0
Q-80	91	6.6	.18	12-101	65	.0	.0
Q-227	80	3.7	.0	13-47	83	3.5	.0
Q-231	68	1.4	.0				

[a] Checks not inoculated.

TIME OF INOCULATION

Inoculations were made at three different stages of anthesis: (1) When the stamens were green, (2) when they were yellow, and (3) when the pollen was being shed. In all cases the smut was placed directly on the stigma with forceps. Data on the time of inoculation are given in table 4. Student's pairing method (*3*) was used to determine whether there were any significant differences. It is obvious from the probability, as shown by *P* in these tables, that there are no significant differences; at least, if there are any, they are covered up by the differential reaction of the varieties.

TABLE 4.—*Percentage of loose-smut infection obtained on different varieties and strains inoculated at two different stages of anthesis and the probability of a difference in the two means*

GREEN AND YELLOW STAMENS

Wheat variety or strain	Percentage of infection when plants were inoculated while stamens were—		Wheat variety or strain	Percentage of infection when plants were inoculated while stamens were—	
	Green	Yellow		Green	Yellow
Alcalde	100.0	100.0	R–S 4–5	77.8	76.9
Onoas	95.0	66.7	R–S–17	77.8	100.0
Sevier 125	60.0	80.0	11–30	70.0	[a] 80.0
1–46	73.7	90.5	Dicklow No. 3	83.3	64.4
Q–248	50.0	60.0	Federation	80.0	[b] 79.80
II–R–18–5	80.0	88.9			
R–48–22	63.6	60.6	Mean	75.9	79.0

STAMENS GREEN AND STAMENS SHEDDING POLLEN

	Green	Shedding pollen		Green	Shedding pollen
Marquis	33.3	33.3	Dicklow No. 3	83.3	[c] 50.0
Q–227	81.8	30.8	Federation	80.0	[d] 89.6
9–7	87.5	78.6			
14–61	22.2	31.6	Mean	64.7	52.3

STAMENS YELLOW AND STAMENS SHEDDING POLLEN

	Yellow	Shedding pollen		Yellow	Shedding pollen
01–24	81.2	81.2	Dicklow No. 3	64.4	[e] 50.0
Q–89	90.9	65.0	Federation	79.8	[f] 89.6
F–68	36.4	52.9			
11–12	81.8	25.0	Mean	73.1	66.2
11–88	76.9	100.0			

[a] $t=0.71$.   [b] $P=0.5$.   [c] $t=1.23$.   [d] $P=0.2-0.3$.   [e] $t=0.65$.   [f] $P=0.5-0.6$.

The data presented in table 4 at first seemed to indicate that more infection was obtained when the stamens were green, as was stated by Tapke (*16*). However, when the data were analyzed statistically, no significant difference was noted between the three periods of inocula-

tion. Maddox (*11*) states that the time of maximum infection is during the period when the pollen is being shed. Freeman and Johnson (*4*) concluded that maximum infection occurs during the period of full bloom and that some degree of infection occurs until the ovary has reached one third its mature size.

STUDIES ON INHERITANCE OF RESISTANCE TO LOOSE SMUT

DIFFICULTIES IN PLACING RESISTANCE ON A DEFINITE FACTORIAL BASIS

Several difficulties are encountered in attempting to place on a definite factorial basis the inheritance of resistance to loose smut, as well as to any other disease. One of the most complicating factors is the effect of environment. This effect was partly reduced by replication. Some comparatively susceptible types occasionally escape the disease when grown in short rows, even though artificially inoculated. For example, Preston on the average smuts about 1.6 percent, with some rows smutting as high as 9.5 percent; however, 70 percent of the rows, in these studies with 30 seeds sown in each row, entirely escaped infection.

The possibility of the inoculum not being entirely uniform, because of the possible existence of physiologic forms of loose smut, also adds to the difficulty of placing the inheritance of resistance on a definite factorial basis. In spite of these complications, an attempt has been made to place resistance on a Mendelian basis.

RELATION OF INFECTION TO SHEATH COLOR

An interesting condition developed on the sheaths and the exposed culms of inoculated plants. Usually the plants with smutty spikes developed a distinct grayish-purple color on the leaf sheaths. At first it appeared as if the coloration were a characteristic of the culm; on closer examination it was found that the coloration was generally confined to the sheath portion of the leaf. It was also found on the portion of the culms exposed to the light. This peculiar coloration developed on Hope and Preston during the year they were inoculated, whereas on Federation no color developed even though the plants smutted. In the F_3 generation there appeared to be a segregation for this condition, suggesting that it may partly be controlled by genetic factors. According to Heald (*7, pp. 683–684*), a similar condition was observed by McAlpine, who stated that "when a stool is affected with loose smut, the stalks are generally of a purplish tint, so that they can be readily picked out from among the general crop."

BIOMETRICAL STUDIES

RELATION OF SMUT INFECTION AND SEEDLING MORTALITY

In the study of disease resistance where the disease organism is operative during the seedling stage of the host plant, it is important to know whether or not there is any differential relationship between the infection and seedling mortality among resistant and susceptible lines. If such a relationship existed, it would no doubt materially complicate a genetic interpretation of inheritance. In order to determine whether this condition did exist, a known number of kernels were seeded in each F_3 row. This made it possible to calculate the

percentage of seedling plants reaching maturity, or the percentage stand. It appeared evident that if the disease were causing the death of any appreciable number of susceptible seedlings, there should be a relationship in the percentage of smut obtained in the F_3 rows and the percentage stand. Simple correlation coefficients were used to measure whether or not a relationship existed; since F_3 rows were replicated, an average of the replication was taken for both the percentage of smut and the percentage of stand; this average replication was used in calculating the correlations. Correlation coefficients thus obtained, between the percentage of smut and the percentage of stand for the susceptible parental strain and for the F_3-progeny rows, are shown in the following tabulation:

```
Material:                                                                    r
    Federation _____  0. 09±0. 167
    Dicklow No. 3 _____    . 10±   . 115
    01–24 _____    . 16±   . 096
    Hope × Federation (F₃ rows) _____     . 02±   . 047
    Hope × Dicklow No. 3 (F₃ rows) _____     . 02±   . 039
    Preston × 01–24 (F₃ rows) _____     . 01±   . 039
```

There seems to be no evidence from these data that smut has any differential influence on the percentage stand in the F_3 rows of resistant and susceptible lines. This is further shown in considering the average percentage stand obtained in the F_3 parental rows, since here it is possible to compare the inoculated resistant and susceptible strains. The average percentage stand was: Hope, 65.1; Preston, 54.6; 01–24, 56.2; Dicklow No. 3, 68.5; and Federation, 75.2.

Thus it appears safe to conclude that under the conditions of the experiment, the smut organism had no greater effect on the seedling mortality, on an average, in susceptible than in resistant lines.

DETERMINING THE CLASS INTERVAL

Because of the unknown experimental errors in percentage of infection occurring from single-row plantings, it seemed advisable to make replicate seedings and to determine the size of the errors in the various crosses, and use these results in interpreting the data. The experiments were planned with a view to using Fisher's (*3*) analysis-of-variance method, and the replicates were randomized accordingly. In two of the crosses there was enough seed for three replications, while in the other cross duplicate seedings only were possible. Analysis-of-variance data for the three crosses are given in table 5.

Fisher's (*3*) Z test was made to determine whether or not there was any treatment effect; the value of Z thus calculated is shown at the bottom of the last column of table 5. Since Fisher's (*3*) tables do not give the value for n_1 and n_2 as occurring in the analysis-of-variance tables, it was necessary to calculate this value from the formula given by him. This value is shown at the bottom of the table. The Z quantity, calculated by comparing the variance due to treatment with that due to error, as shown in table 5, is larger in all cases than Z for the 1-percent point. This shows that there is undoubtedly a treatment effect, which naturally was to be expected from the nature of the material, since the mean percentage of smut occurring in the replicated F_3 rows ranged from no smut to a rather high percentage.

TABLE 5.—*Analysis of variance for three wheat crosses*

HOPE × FEDERATION CROSS

Variance due to—	Degrees of freedom	Sum of squares	Mean square	Half log
Replication	1	0.03		
Treatment	206	95,176.77	462.02	3.0678
Error	206	11,459.99	55.63	2.0090
Total	413	106,636.79		$Z=1.0584$

$n_1=206$ $n_2=206$ Z−1-percent point=0.1625

PRESTON × 01-24 CROSS

Replication	2	571.09		
Treatment	306	39,743.41	129.88	2.4335
Error	614	44,335.41	72.44	2.1414
Total	922	116,636.79		$Z=.2937$

$n_1=306$ $n_2=614$ Z−1-percent point=0.1148

HOPE × DICKLOW

Replication	2	149.91		
Treatment	306	78,482.39	256.48	2.7736
Error	614	37,498.94	61.27	2.0579
Total	922	116,131.24		$Z=.7157$

$n_1=306$ $n_2=614$ Z−1-percent point=0.1146

It is evident from table 5 that the variance of a single determination is 55.63 for the Hope × Federation cross, 72.44 for the Preston × 01–24 cross, and 61.27 for the Hope × Dicklow No. 3 cross. These values were used in each case to set up a difference necessary between two mean percentages of infection for a probability of 0.05. Since the error was based on a rather large number, a significant difference would amount to about twice the standard error of the difference. This quantity amounted to about 13 percent in the Hope × Dicklow No. 3 cross, 14 percent in the Preston × 01–24 cross, and 15 percent in the Hope × Federation cross. Then, in classifying the F_3 rows, the value necessary for a significant difference was taken as the class interval; on this basis a frequency table was constructed. The proportion of F_3 rows falling into each of the classes and the reaction of the parental types formed the basis for arriving at the factorial explanation for the inheritance of resistance to loose smut herein suggested.

GENETIC STUDIES OF RESISTANCE TO LOOSE SMUT

The reaction of the parental material to loose smut (*Ustilago tritici*), as shown in table 6, seemed to indicate that possibly more than one factor was involved in the inheritance of resistance. This was also suggested by the breeding behavior of the F_3 progeny of the various crosses. On the basis of these two conditions, it was assumed that at least three factors were involved in the inheritance of resistance to loose smut. This factorial relationship of the parental material is shown in table 6.

Since Hope has never smutted even though thousands of inoculated plants have been grown, it was considered to be completely immune to the inoculum used; therefore, it possessed all three factors in the dominant condition, though dominance is evidently incomplete and the factors have a cumulative effect. Likewise, each of the three factors are thought to have a different effect, an individual with the R_2R_2 factor showing somewhat more resistance than one with the R_3R_3 factor and one with the R_1R_1 factor being about as resistant as one possessing the other two factors. This does not mean that these factors have definite numerical values with specific expression, regardless of the genotype, as factor interaction is not an uncommon phenomenon.

On an average, Preston has smutted about 1.6 percent, although when it is grown in rows of approximately 30 seeds to the row, similar to the F_3-progeny rows, about 70 percent of the rows show no smut at all; other rows show as much as 9.5 percent smut. Consequently, it was assumed that Preston lacked one of the factors common to Hope and that the absence of this factor allows some smut to develop. Preston would then be classed as highly resistant but not immune, as was Hope.
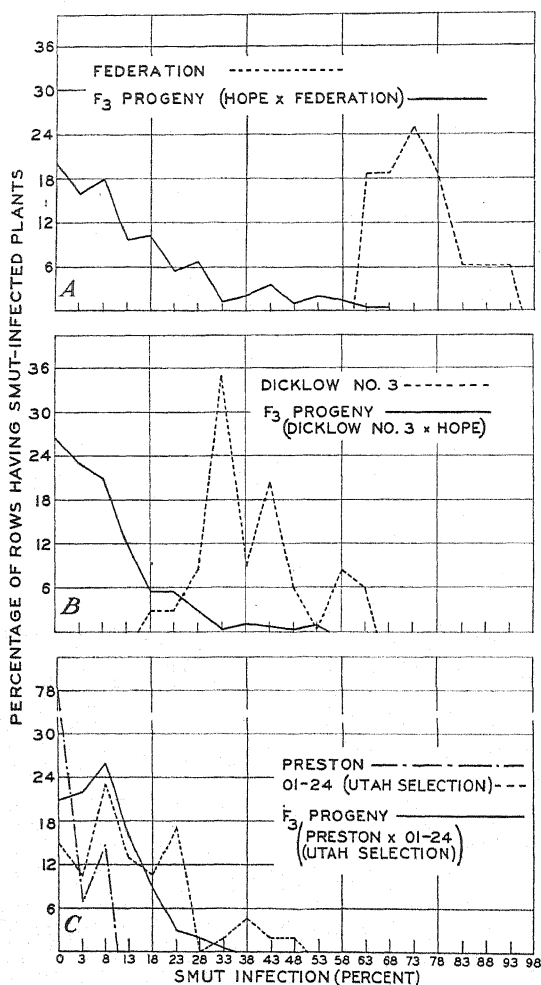
FIGURE 3.—Frequency distribution of parental varieties and F₃ progeny in various wheat crosses as related to loose-smut infection. *A*, Federation and F₃ progeny of Hope × Federation; *B*, Dicklow No. 3 and F₃ progeny of Dicklow No. 3 × Hope; *C*, Preston, 01–24 (Utah selection) and F₃ progeny of Preston × 01-24 (Utah selection).

Under similar conditions, 01–24 smuts about 20 percent. Therefore, it was assumed that it possesses two factors in common with Hope but that it differs from Preston in two factors.

Dicklow No. 3 apparently was more susceptible than 01–24 and, therefore, was assigned only the one factor, R_3R_3; lacking R_1R_1 and

R_2R_2, it is comparatively susceptible and smuts on an average nearly 40 percent.

TABLE 6.—*Genetic composition assigned each of the parental types, on the basis of reaction to loose smut, the range, and the average percentage of infection occurring in rows of 30 kernels each*

Parent	Genotype	Rows	Infection	
			Range	Average
		Number	*Percent*	*Percent*
Hope C.I. 8178	$R_1R_1\ R_2R_2\ R_3R_3$	50	0–0	0
Preston C.I. 3081	$R_1R_1\ R_2R_2\ r_3r_3$	26	0–9.5	1.6±0.39
01–24 C.I. 11542	$r_1r_1\ R_2R_2\ R_3R_3$	47	0–50.0	18.5±1.21
Dicklow No. 3	$r_1r_1\ r_2r_2\ R_3R_3$	34	18.2–61.9	38.5±1.27
Federation	$r_1r_1\ r_2r_2\ r_3r_3$	16	60.9–91.3	73.5±1.57

Federation is assumed to possess none of the factors for resistance, since it ordinarily smuts 70 percent or more.

In table 7 is shown the distribution of the parents and the F_3 of the various crosses in 5-percent classes for loose-smut infection, and figure 3 is a graphical presentation of the same data.

HOPE × FEDERATION CROSS

It is evident from the factorial relationship assigned each parental type that the Hope × Federation cross should give rise to 27 F_2 genotypes. The reaction of the known genotypes, the parents, to the smut inoculum is shown in table 6. On the basis of these known types, the behavior of the remaining genotypes was formulated.

TABLE 7.—*Distribution of parents and F_3 rows of the crosses named in 5-percent classes for loose-smut infection*

Parent or cross	Rows smut-free	Rows having percentages of loose-smut infection falling within the indicated 5-percent classes (average of three replications for F_3; single rows for parents)																			Total rows
		3 [a]	8	13	18	23	28	33	38	43	48	53	58	63	68	73	78	83	88	93	
Hope C.I. 8178 number	16																				16
Federation number	0													3	3	4	3	1	1	1	16
91b-Hope (C.I. 8178) × Federation number	43	33	38	20	22	12	13	3	4	7	3	4	3	1	1						207
Hope C.I. 8178 number	34																				34
Dicklow No. 3 (Utah selection) number	0				1	1	3	12	3	7	2	0	3	2							34
87b-Hope C.I. 8178 × Dicklow No. 3 (Utah selection) number	82	71	65	36	17	17	8	3	4	1	0	2									307
Preston C.I. 3081 number	20	2	4																		26
01–24 (Utah selection) number	7	5	11	6	5	8	0	1	2	1	1										47
93b-Preston × 01–24 (Utah selection) number	65	71	81	46	27	9	7	1													306

[a] Taken to the nearest whole number.

The basic phenotypic ratio ordinarily obtained in F_2 in a cross, when three independent factors expressing different characters are involved, is 27 : 9 : 9 : 9 : 3 : 3 : 3 : 1. This ratio, however, may be modified by factor interaction. In the case under consideration the factors were assumed to be of unequal value and were all involved in the expression of a single character. It has been shown that strains even though somewhat susceptible, escape infection when sown in short rows and, therefore, would be placed in the nonsmutting group.

This would tend to make this group too large. To obviate this difficulty, those progenies showing no infection were included in the lowest frequency group. This class interval permitted a certain percentage of infection, which in this cross included those with no infection up to 14.9 percent. The method of arriving at the class interval has previously been discussed. The reason for using the experimental error in arriving at the class interval is that F_3 strains may actually be susceptible up to as much as 15 percent, and yet a certain proportion may escape infection when grown in short rows even though replicated. This would also be true if the interval were extended, but not with the same probability. The phenotypes, the theoretical parental types, and the basis of classification of the F_3 rows in the Hope $\times$ Federation cross are shown in table 8.

TABLE 8.—*Possible F_2 genotypes; the theoretical parental type; the percentage of infection, and the basis of classification of the F_3; also the phenotypic ratio in the Hope $\times$ Federation cross*

Genotype	Number of each	Theoretical parental type	Actual parental average infection	F_3 classification Class interval	F_3 classification Average infection [a]	F_3 classification Phenotypic ratio
$R_1R_1\ R_2R_2\ R_3R_3$	1					
$R_1R_1\ R_2R_2\ R_3r_3$	2					
$R_1R_1\ R_2r_2\ R_3R_3$	2					
$R_1r_1\ R_2R_2\ R_3R_3$	2	Hope	*Percent* 0	*Percent*	*Percent*	
$R_1R_1\ R_2r_2\ R_3r_3$	4					
$R_1r_1\ R_2R_2\ R_3r_3$	4					
$R_1r_1\ R_2r_2\ R_3R_3$	4					
$R_1r_1\ R_2r_2\ R_3r_3$	8			0–14.9	6.9	
Total	27					
$R_1R_1\ R_2R_2\ r_3r_3$	1					
$R_1R_1\ R_2r_2\ r_3r_3$	2	Preston	1.6			45
$R_1r_1\ R_2R_2\ r_3r_3$	2					
$R_1r_1\ R_2r_2\ r_3r_3$	4					
Total	9					
$R_1R_1\ r_2r_2\ R_3R_3$	1					
$R_1R_1\ r_2r_2\ R_3r_3$	2	Intermediate (Preston and 01–24) [b]				
$R_1r_1\ r_2r_2\ R_3R_3$	2					
$R_1r_1\ r_2r_2\ R_3r_3$	4					
Total	9					
$r_1r_1\ R_2R_2\ R_3R_3$	1					
$r_1r_1\ R_2R_2\ R_3r_3$	2	01–24	18.5	15–29.9	19.9	
$r_1r_1\ R_2r_2\ R_3R_3$	2					
$r_1r_1\ R_2r_2\ R_3r_3$	4					12
Total	9					
$R_1R_1\ r_2r_2\ r_3r_3$	1	Equal to 01–24 [c]	18.5			
$R_1r_1\ r_2r_2\ r_3r_3$	2					
Total	3					
$r_1r_1\ R_2R_2\ r_3r_3$	1	Intermediate (01–24 and Dicklow No. 3) [d]		30–44.9	34.9	3
$r_1r_1\ R_2r_2\ r_3r_3$	2					
Total	3					
$r_1r_1\ r_2r_2\ R_3R_3$	1	Dicklow No. 3	38.5	45–59.9	46.5	3'
$r_1r_1\ r_2r_2\ R_3r_3$	2					
Total	3					
$r_1r_1\ r_2r_2\ r_3r_3$	1	Federation	73.5	60–74.9	62.3	1

[a] The average of the class and not the mid point.
[b] Not as resistant as Preston but more resistant than 01–24; somewhere between 1.6 and 18.5 percent.
[c] The R_1R_1 factor was assumed to be equivalent in effect to the R_2R_2 and R_3R_3 factors.
[d] Not as resistant as 01–24 but more resistant than Dicklow No. 3, somewhere between 18.5 and 38.5 percent.

On this basis, Hope, Preston, and the Intermediate (Preston and 01–24) genotypes all fell in the same class. It is evident from table 8 that forty-five sixty-fourths of the F_3 progeny rows would theoretically fall in the first phenotypic group, in which infection would range from 0 to 14.9 percent. In the second group infection ranged from 15 to 29.9 percent; this group included the genotypes corresponding to the 01–24 parental type. Inasmuch as the R_1R_1 factor carried by Hope is assumed to be equal in effect to the other two factors, twelve sixty-fourths of the progeny rows would be expected in the second-class interval. In the third group infection ranged from 30 to 44.9 percent. This group was composed of the genotypes which were neither 01–24 nor Dicklow No. 3 types but which fell somewhere between them; three sixty-fourths of the progeny rows should be of this type. The genotypes corresponding to the Dicklow No. 3 parental type constituted the fourth group, in which infection ranged from 45 to 59.9 percent; three sixty-fourths of the progeny rows would also be expected to be in this class. Infection in the last or upper group ranged from 60 to 74.9 percent; this group included the genotype characteristic of Federation and included only one sixty-fourth of the progeny rows. This completes the phenotypic ratio of 45 : 12 : 3 : 3 : 1. Table 9 shows the goodness of fit obtained when the observed data were fitted to this ratio. The probability is between 0.2 and 0.3 and is considered satisfactory.

TABLE 9.—*Goodness of fit obtained from the breeding behavior in F_3 in regard to loose-smut resistance in the Hope* × *Federation cross, based on a 45 : 12 : 3 : 3 : 1 ratio*

Smut (percent)	Number of progeny	
	Observed	Calculated
0 to 14.9	132	143. 0
15 to 29.9	43	39. 4
30 to 44.9	14	9. 6
45 to 59.9	13	9. 6
60 to 74.9	5	3. 2

$x_2 = 5.4085$.
$P = 0.2 - 0.3$.

HOPE × DICKLOW NO. 3

It will be observed that Hope and Dicklow No. 3 differ from each other in two factors and that there will be no genotypes which do not carry the R_3R_3 factor for resistance. The possible F_2 genotypes, the theoretical parental types, the basis of classification of the F_3 rows, and the phenotypic ratio of Hope × Dicklow No. 3 cross are shown in table 10.

TABLE 10.—*Possible F₂ genotypes, the theoretical parental type, percentage infection, and the basis of classification of F₃, also the phenotypic ratio in the Hope ✕ Dicklow No. 3 cross*

Genotype	Number of each	Theoretical parental type	Actual parental average infection	F₃ classification		
				Class interval	Average infection[a]	Phenotypic ratio
R_1R_1 R_2R_2 R_3R_3 ------	1	} Hope _____	*Percent* 0	*Percent* 0-12.9	*Percent* 6.8	} 12
R_1R_1 R_2r_2 R_3R_3 --------	2					
R_1r_1 R_2R_2 R_3R_3 --------	2					
R_1r_1 R_2r_2 R_3R_3 ---------	4					
Total _____	9					
R_1R_1 r_2r_2 R_3R_3 ---------	1	} Intermediate (Preston and 01-24)[b].	----------	----------	----------	
R_1r_1 r_2r_2 R_3R_3 ---------	2					
Total _____	3					
r_1r_1 R_2R_2 R_3R_3 ---------	1	} 01-24 _____	18.5	13-25.9	17.1	3
r_1r_1 R_2r_2 R_3R_3 ---------	2					
Total _____	3					
r_1r_1 r_2r_2 R_3R_3 ---------	1	Dicklow No. 3 _____	38.5	26-55.0	37.1	1

[a] The average of the class and not the mid point.
[b] Not as resistant as Preston but more resistant than 01-24; somewhere between 1.6 and 18.5 percent.

The amount necessary to give a significant difference between two mean percentage infections, calculated as previously stated (with a probability of 0.05) was approximately 13 percent. Consequently, this was the amount used as the class interval in separating the phenotypic groups and in determining the phenotypic ratio. As in the Hope ✕ Federation cross, no attempt was made to differentiate between the genotypes resembling the Hope parent and those which carried the R_1R_1 and R_3R_3 factors, making them intermediate (Preston and 01-24) types, because they were all included in the same class interval. The range of smut infection allowed in the first phenotypic group was from 0 to 12.9 percent. The class interval in this cross is slightly less than that allowed for the corresponding genotypes of the Hope ✕ Federation cross. This reduction in class interval was due to the slight difference in the variance obtained in this cross and also to the fact that there were triplicate plantings which would reduce the standard error of a difference accordingly. Inasmuch as the class interval is reduced 2 percent in this cross, the range of smut allowed by the various genotypes is correspondingly reduced. Infection in the second phenotypic group ranged from 13 to 25.9 percent and included the genotypes typified by the 01-24 parent. The upper class included all strains with 26 percent infection or more. There were only 20 of the 307 progeny rows that smutted above that amount. This was almost the exact number expected to conform to the Dicklow No. 3 genotype. Infection in the progeny included in this group ranged from 27.1 to 55 percent and averaged 37.05. This is about what would be expected of plants having a genetic make-up equivalent to Dicklow No. 3. For this reason all strains with 26-percent infection or over were considered as one class. This classification gave a phenotypic ratio of 12 : 3 : 1. Table 11 shows the goodness of fit obtained when the observed data were compared with the above ratio. It is evident from table 11 that the goodness of fit was again satisfactory.

TABLE 11.—*Goodness of fit obtained from the breeding behavior in F_3 in regard to loose-smut resistance in the Hope × Dicklow No. 3 cross, based on a 12:3:1 ratio*

	Number of progeny	
Smut (percent)	Observed	Calculated
to 12.9	236	230. 2
13 to 25.9	50	57. 6
Above 26	20	19. 2

$\chi_2 = 1.2169$.
$P = 0.5–0.7$.

In the Preston × 01–24 cross all the factors common to Hope are involved. However, the R_2R_2 factor is present in both parents; as a result, this factor appears in a homozygous dominant condition in all the genotypes obtained from the cross. Data relative to this cross are shown in table 12.

TABLE 12.—*Possible F_2 genotypes, the theoretical parental type, the percentage of infection, and the basis of classification of the F_3, as well as the phenotypic ratio in the Preston × 01-24 cross*

Genotype	Number of each	Theoretical parental type	Actual parental average infection	F₃ classification		
				Class interval	Average infection[a]	Phenotypic ratio
$R_1R_1\ R_2R_2\ R_3R_3$	1		*Percent*	*Percent*	*Percent*	
$R_1R_1\ R_2R_2\ R_3r_3$	2	Hope	0	0–13. 9	5. 8	
$R_1r_1\ R_2R_2\ R_3R_3$	2					
$R_1r_1\ R_2R_2\ R_3r_3$	4					12
Total	9					
$R_1R_1\ R_2R_2\ r_3r_3$	1	Preston	1. 6			
$R_1r_1\ R_2R_2\ r_3r_3$	2					
Total	3					
$r_1r_1\ R_2R_2\ R_3R_3$	1	01–24	18. 5	14–27. 9	16. 9	3
$r_1r_1\ R_2R_2\ R_3r_3$	2					
Total	3					
$r_1r_1\ R_2R_2\ r_3r_3$	1	Intermediate[b] (01–24 and Dicklow No. 3.)		28–41. 9	32. 0	1

[a] Average of the class and not the mid point.
[b] Not as resistant as 01–24 but more resistant than Dicklow No. 3; somewhere between 18.5 and 38.5 percent.

PRESTON × 01-24

In conformity with the two previously discussed crosses, no distinction was drawn between the genotypes resembling the Hope parent and those resembling the Preston parent, because the range of smut allowed by each came within the range of the class interval. Thus, twelve sixty-fourths of the progeny rows were included in the first phenotypic group. The second group, ranging in infection from 14 to 27.9 percent, was made up of the genotypes resembling the 01–24 parent. Only 10 of the 307 F_3 rows smutted above 28 percent. These ranged in infection from 28 to 39 percent, with an average of 32, which is about as expected since the genotype of this phenotypic

class carries the R_2R_2 factor, which should make it an intermediate (01–24 and Dicklow No. 3) type. From this classification a 12 : 3 : 1 ratio was expected; the goodness of fit obtained when the observed was compared with this ratio is shown in table 13.

TABLE 13.—*Goodness of fit obtained from the breeding behavior in F_3 in regard to loose-smut resistance in the Preston $\times$ 01–24 cross based on a 12 : 3 : 1 ratio*

Smut (percent)	Number of progeny	
	Observed	Calculated
0 to 13.9	237	230. 2
14 to 27.9	60	57. 6
Above 28	10	19. 2

$\chi^2 = 4.7092$.
$P = 0.05-0.1$.

It is evident from table 13 that while the fit is not exceptionally good, nevertheless it is within the lower limits of probability usually set (0.05).

FACTORIAL RELATIONSHIP OF THE VARIOUS CROSSES

The fact that the same factors for resistance were assumed to be involved in all three crosses suggests that the progeny of similar genetic constitution obtained from the various crosses ought to give a similar reaction to the inoculum. This relationship is also shown in tables 8, 10, and 12. Infection in the phenotypic class of 45 in the Hope $\times$ Federation cross (table 8) ranged from 0 to 14.9 percent, with an average of 6.9; the corresponding class of 12 in the Hope $\times$ Dicklow No. 3 cross (table 10) ranged in infection from 0 to 12.9 and averaged 6.7; and the class of 12 in the Preston $\times$ 01–24 cross (table 12) ranged in infection from 0 to 13.9, with an average of 5.8. There is a rather close agreement in the mean percentages of infection in the three crosses for the lower class. The Preston $\times$ 01–24 cross should show a lower mean infection in the lower class because there were no intermediate (Preston and 01–24) types present. These types appearing in the other two crosses should be more susceptible than the Preston type. It will be noted (table 8, 10, and 12) that the average percentage of infection for the lower phenotypic group in all three crosses is slightly higher than for the Preston parent, which is most typical of the genotypes included in this class. This is to be expected in the Hope $\times$ Federation and the Hope $\times$ Dicklow No. 3, crosses, however, because the class interval is extended beyond that for Preston, and, therefore, includes intermediate (Preston and 01–24) types, which would be more susceptible than Preston; this would account for the higher average. The average of the lower class in the Preston $\times$ 01–24 cross is higher than expected. However, this does not appear especially serious as the same exactness in a study of disease resistance cannot be expected as in a study of morphological characters. This discrepancy may be due to any one or to a combination of the four following conditions:

(1) The heterozygous condition of some of the genotypes, which may allow for more infection than the homozygous, since dominance is not complete.

(2) The effect of modifying factors.

(3) The differential infection in parent and in F_3 progeny due to slight differences in the stage of inoculation. Although instructions were given to inoculate both parent and progeny before or soon after the anthers had shed their pollen (inoculations at later stages appears to reduce materially the amount of infection), it might have been possible that a number of Preston spikes were inoculated somewhat later, when the kernels were partly formed. This would lower the average percentage of infection of Preston. In this connection it might be well to mention that in 1933 Preston showed 9.5 percent of infection when inoculated with the same smut. It is not known whether this was due to differences in stages of inoculation or to soil and climatic differences.

(4) It is possible that one of the parents, especially Preston, was a mixed population. Therefore, the pure line of Preston used in this cross may have been slightly more susceptible than the average for the variety. This would necessitate a slight change in the genetic constitution of Preston from $R_1R_1R_2R_2r_3r_3$ to one of a type $R_1R_1r_2r_2R_3R_3$ since the R_3R_3 factor allows more infection than the R_2R_2 factor. One would now expect the theoretical average of the first class to be considerably above 1.6 percent.

In view of these various possibilities, it appears desirable to leave the genetic composition of the varieties as shown in table 6.

The average percentage of infection for genotypes similar to 01–24 and Dicklow No. 3, in all crosses was within a few percent of these parental types (tables 8, 10, and 12). In the Hope × Federation cross (table 8) the 45 progeny observed in the upper class had an average of infection lower than that of the Federation parent, although they were within the range of the Federation; with the few progeny rows the differences may be due purely to error in sampling. In the Hope × Dicklow No. 3 cross no types were recovered with any higher range of infection than that of the Dicklow No. 3 parent. The 20 rows falling in this class had an average infection of 37.1 as compared with 38.5 for Dicklow No. 3. The Preston × 01–24 cross gave no genotypes which did not carry the R_2R_2 factor; hence, the highest infection expected was somewhere between the range of the parental varieties 01–24 and Dicklow No. 3. This would be somewhere between 18.6 and 38.5 percent. The average actually obtained for this class was 32 percent (table 12). In general, it appears as though the genotypes recovered from the various crosses correspond-ing to the parental types react to loose smut similarly to the parental types used and as though the similar genotypes recurring in the differ-ent crosses behave in a similar manner.

STUDIES ON THE INHERITANCE OF MORPHOLOGICAL CHARACTERS AND THEIR RELATION TO RESISTANCE TO LOOSE SMUT

INHERITANCE OF MORPHOLOGICAL CHARACTERS

The primary purpose in obtaining the breeding behavior of the awns, chaff, and kernel color was to determine whether or not there was any relationship between any of these morphological characters and disease resistance. The knowledge of such a relationship, if it did exist, would be of great value to the plant breeder.

and calculated numbers falling in each class and the goodness of fit based on a 1:2:1 ratio. It is apparent from table 14 that a good fit was obtained in both crosses.

TABLE 14.—F_3 breeding behavior of awns in the Hope × Dicklow No. 3 and the Preston × 01-24 crosses, and the goodness of fit based on a 1:2:1 ratio

Parent [a]	Class	Observed	Calculated	x^2	P
Hope (*AABB*) × Dicklow No. 3 (*aaBB*).	Short apical awns	82	76.7		. 0.7–0.8
	Segregating	150	153.3	0.4890	
	Fully awned	75	76.7		
Preston (*AABB*) × 01-24 (*aaBB*)	Short apical awns	78	76.7		
	Segregating	158	153.4	.5835	.7–.8
	Fully awned	71	76.7		

[a] Awnedness was arbitrarily chosen to be represented by the dominant characters; awnlessness might just as appropriately be so designated.

HOPE × FEDERATION CROSS.—The inheritance of awns in the Hope × Federation cross was quite different from that in the Hope × Dicklow No. 3 and Preston × 01-24 crosses just discussed. The F_1 in the Hope × Federation cross was intermediate in inheritance but again resembled most closely the awnless parent. The parental types, the F_1, and the true-breeding F_3 types of the Hope × Federation cross are shown in figure 5. Besides the four homozygous types shown, there were five segregating classes of progeny: (1) Those segregating for awn classes 1 and 2; (2) those segregating for awn classes 1,2, and 3; (3) those segregating for awn classes 1, 2, 3, and 4; (4) those segregating for awn classes 2, 3, and 4; and (5) those segregating for awn classes 3 and 4. There were, therefore, nine genotypic classes into which the F_3 progenies were classified. This breeding behavior suggested a two-factor difference with independent segregation. The relation of the observed to the calculated based on a two-factor difference and the closeness of fit to a 1:2:2:4:1:2:1:2:1 ratio is shown in table 15. It is evident from this table that a good fit was obtained.

TABLE 15.—Breeding behavior of awns in the Hope × Federation cross and the goodness of fit to a 1:2:2:4:1:2:1:2:1 ratio

F_3 breeding behavior	Observed	Calculated	x^2	P
True breeding 4	15	13.0		
Segregating 3, 4	20	26.0		
Segregating 2, 3, 4	25	26.0		
Segregating 1, 2, 3, 4	50	52.0		
True breeding 3	16	13.0	3.3077	0.90–0.95
Segregating 1, 2, 3	25	26.0		
True breeding 2	14	13.0		
Segregating 1, 2	26	26.0		
True breeding 1	16	13.0		

KERNEL COLOR

Kernel-color inheritance was involved in all three crosses. The F_1 plants all had red grain and segregation took place in F_2.

HOPE × FEDERATION AND HOPE × DICKLOW No. 3 CROSSES.—The proportion of white to red kernels in the F_2 in the Hope × Federation and Hope × Dicklow No. 3 suggested a three-factor difference, with each factor either alone or in combination expressing the character.

In the Hope × Dicklow No. 3 cross 5 plants out of 307 had white grain; it was assumed to be similar to the Hope × Federation cross, which had 4 white-kerneled plants out of 206 F_2's.   Therefore, the Hope × Dicklow No. 3 cross was not studied for grain color in the F_3.

Studies made on the inheritance of kernel color in F_3 in the Hope × Federation cross behaved as would be expected from a study of the F_2 data.   With a three-factor difference, the F_3 should theoretically segregate into 5 classes, giving a 37 : 12 : 8 : 6 : 1 ratio.   The five classes into which the F_3's were classified, based on kernel color, are shown in table 16.   In this table is also shown the observed number in each class which were fitted to a 37 : 12 : 8 : 6 : 1 ratio with a good fit resulting, as shown by the x^2 test.

TABLE 16.—*Breeding behavior for grain color in the Hope × Federation cross and the goodness of fit based on a 37 : 12 : 8 : 6 : 1 ratio*

Class	Number of progeny		x^2	P
	Observed	Calculated		
True-breeding red	122	118.4		
Segregating 15:1	36	38.4		
Segregating 63:1	23	25.6	0.7941	0.9–0.95
Segregating 3:1	21	19.2		
True-breeding white	4	3.2		

PRESTON × 01–24 CROSS.—Kernel-color studies in the F_2 generation in the Preston × 01–24 cross suggested a single-factor difference. The segregation in F_3 substantiated the results of the findings in the previous generation.   Three classes were observed in F_3.   The proportion of the F_3 rows falling in each of these classes is shown in table 17.   The x^2 test shows a good fit to the expected 1 : 2 : 1 ratio.

TABLE 17.—*Breeding behavior for grain color in the Preston × 01–24 cross and the goodness of fit based on a 1 : 2 : 1 ratio*

Class	Number of progeny		x^2	P
	Observed	Calculated		
True-breeding white	82	76.7		
Segregating	149	153.4	0.5039	0.7–0.8
True-breeding red	76	76.7		

GLUME COLOR

HOPE × FEDERATION AND PRESTON × 01–24.—The F_2 data on the two crosses, Hope × Federation and Preston × 01–24, indicated that in each case there was a single-factor difference for chaff color.   In the Hope × Dicklow No. 3 cross both parents had white chaff, and thus no segregation occurred.   Table 18 shows the F_3 breeding behavior of the two crosses for chaff color and the goodness of fit based on a 1 : 2 : 1 ratio.   The x^2 test shows a good fit in both crosses.

TABLE 18.—_Breeding behavior for glume color in the Hope $\times$ Federation and Preston $\times$ 01–24 crosses and the goodness of fit based on a 1 : 2 : 1 ratio_

Parent	Class	Number of progeny		χ^2	P
		Observed	Calculated		
Hope $\times$ Federation	True-breeding white	56	51.7	0.7032	0.7–0.8
	Segregating	99	103.4		
	True-breeding bronze	52	51.7		
Preston $\times$ 01–24	True-breeding white	76	76.7	.4988	.7–0.8
	Segregating	149	153.4		
	True-breeding bronze	82	76.7		

RELATION OF MORPHOLOGICAL CHARACTERS AND RESISTANCE TO LOOSE SMUT

In order to determine whether a relationship exists between morphological characters and resistance to loose smut, a series of contingency tables was prepared showing a comparison of the reaction to smut infection and morphological characters. A measure of the relationship between the distribution of the two characters being compared may be obtained by calculating χ^2 and determining the value of P from Fisher's (3) tables. Table 19 shows the distributions and the probability obtained in each case. Table 20 gives a summary of all comparisons. In interpreting results it is safe to assume that if the value of P for any given distribution is higher than 0.05, there is no evidence of significant correlation between the characters being considered.

TABLE 19.—_Contingency table for grain color, chaff color, and awns and smut classes as occurring in the F_3 progeny of various crosses_

GRAIN COLOR AND SMUT CLASSES IN PRESTON $\times$ 01–24 CROSS

Smut (percent)	Number of progeny				χ^2	P
	White	Segregating	Red	Total		
0 to 13.9	59	114	64	237		
14 to 27.9	15	29	16	60		
28 to 41	2	6	2	10	0.5489	0.2–0.3
Total	76	149	82	307		

GRAIN COLOR AND SMUT CLASSES IN THE HOPE $\times$ FEDERATION CROSS

	White	Segregating	Red	Total	χ^2	P
0 to 14.9	1	51	82	134		
15 to 29.9	2	15	24	41		
30 to 44.9	1	7	6	14		
45 to 59.9	0	5	8	13	6.4055	0.5–0.7
60 to 74.9	0	2	3	5		
Total	4	80	123	207		

CHAFF COLOR AND SMUT CLASSES IN THE PRESTON $\times$ 01–24 CROSS

Smut (percent)	Number of progeny				χ^2	P
	White	Segregating	Bronze	Total		
0 to 13.9	61	116	60	237		
14 to 27.9	13	27	20	60	2.1727	0.7–0.8
28 to 41.9	2	6	2	10		
Total	76	149	82	307		

TABLE 19.—*Contingency table for grain color, chaff color, and awns and smut classes as occurring in the F_3 progeny of various crosses*—Continued

CHAFF COLOR AND SMUT CLASSES IN THE HOPE × FEDERATION CROSS

Smut (percent)	Number of progeny				χ^2	P
	White	Segregating	Bronze	Total		
0 to 14.9	38	65	29	132		
15 to 29.9	9	24	10	43		
30 to 44.9	6	4	4	14		
45 to 59.9	2	5	6	13		
60 to 74.9	1	1	3	5		
Total	56	99	52	207	10.8819	0.2–0.3

AWNS AND SMUT CLASSES IN THE PRESTON × 01-24 CROSS

Smut (percent)	Number of progeny				χ^2	P
	No. 1	Segregating	No. 4	Total		
0 to 13.9	62	123	52	237		
14 to 27.9	14	29	17	60		
28 to 41.9	2	6	2	10		
Total	78	158	71	307	1.4196	0.8–0.9

AWNS AND SMUT CLASSES IN THE HOPE × DICKLOW NO. 3 CROSS

	No. 1	Segregating	No. 4	Total	χ^2	P
0 to 12.9	64	113	59	236		
13 to 25.9	13	26	11	50		
26 to 55.0	5	11	5	21		
Total	82	150	75	307	0.4486	0.95–0.98

AWNS AND SMUT CLASSES IN THE HOPE × FEDERATION CROSS

Smut (percent)	Number of progeny					χ^2	P
	No. 1.	No. 2	No. 3	No. 4	Total		
0 to 14.9	7	10	9	10	36		
15 to 29.9	4	2	2	3	11		
30 to 44.9	3	1	3	1	8		
45 to 59.9	1	1	2	1	5		
60 to 74.9	1	0	0	0	1		
Total	16	14	16	15	61	6.9854	0.8–0.9

Since the lowest P value in table 20 is between 0.2 and 0.3, there seems to be no indication of relationship between resistance or susceptibility and any of the morphological characters studied. This is interesting in view of the fact that Fromme (*5, 6*) has reported that awned varieties were more susceptible to loose smut than were awnless varieties. The question arises, was there any relationship between these two characters from the standpoint of inheritance, or did it just happen that those awned varieties with which Fromme was dealing smutted more than the awnless? The studies herein reported indicate that there is no relationship between awns and susceptibility or resistance; in fact, the awned varieties used in these studies were resistant.

Table 20.—*Summary of the χ^2, and P values as given in table 19*

Cross	Characters tested for relationship	χ^2	P
Hope × Federation	Grain color and smut reaction	6. 4055	0. 5 −0. 7
Preston × 01–24	do	.5489	.2 − .3
Preston × 01–24	Chaff color and smut reaction	2. 1727	.7 − .8
Hope × Federation	do	10. 8819	.2 − .3
Preston × 01–24	Awns and smut reaction	1. 4196	.8 − .9
Hope × Dicklow No. 3	do	.4486	.95− .98
Hope × Federation	do	6. 9854	.8 − .9

SUMMARY

Genetic studies on the inheritance of loose-smut resistance, awns, grain color, and chaff color are reported for the Hope × Federation, Hope × Dicklow No. 3, and Preston × 01–24 crosses.

The relative effects of the time and method of inoculation as related to infection are also reported. Maximum infection was obtained only when the smut spores were placed directly on the stigmas. There appeared to be little or no difference in the amount of infection occurring when the plants were inoculated at the time the stamens were rather green and immature and when they were inoculated when the plants were in bloom. In the inheritance studies on loose smut, triplicate randomized plantings were made in two of the crosses and duplicate seedings in the other. This permitted a statistical study of the size of the experimental error. The analysis-of-variance method was used in these studies from the error obtained in the various crosses, it was used to determine a difference which might be considered as significant, on the basis of a probablility of 0.05 between two percentages of infection. The amount obtained was used as the class interval in determining the inheritance of resistance to loose smut. The number of F_3 rows falling within the various class intervals, along with the reaction of the parental material to the disease, formed the basis for the proposed factorial relationship.

The genetic studies of awns and both kernel and chaff color were made in the usual way. The proposed genetic composition of the parental material, for the characters studied, based on their behavior in the previously mentioned crosses is given in table 21.

There was no evidence in the studies made of any relationship between the morphological characters and resistance to loose smut.

Table 21.—*Proposed genetic composition of parental material for characters studied in crosses made*

Variety	Loose smut	Awns	Kernel color	Glume color
Hope	R_1R_1 R_2R_2 R_3R_3	AA BB	K_1K_1 K_2K_2 K_3K_3	gg
Preston	R_1R_1 R_2R_2 r_3r_3	AA BB	K_1K_1 k_2k_2 k_3k_3	gg
01–24	r_1r_1 R_2R_2 R_3R_3	aa BB	k_1k_1 k_2k_2 k_3k_3	GG
Dicklow No. 3	r_1r_1 r_2r_2 R_3R_3	aa BB	k_1k_1 k_2k_2 k_3k_3	gg
Federation	r_1r_1 r_2r_2 r_3r_3	aa bb	k_1k_1 k_2k_2 k_3k_3	GG

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THE MANGANESE CONTENT OF GRASSES AND ALFALFA FROM GRAZED PLOTS [1]

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INTRODUCTION

The nutritive value of manganese and its occurrence in animal and plant tissue has received considerable attention within the last few years. The literature relating to manganese in plant tissue has been reviewed by Lindow and Peterson (6)[3], who reported analyses of 84 samples representing the principal classes of human foods. In a later paper by Skinner and Peterson (12), analyses of 54 feeding stuffs were given, which show a wide variation in the manganese content of plant material. Several other investigators (9, pp. 25–26) have contributed to the knowledge of the occurrence of manganese in foods and feeding stuffs.

In this paper is shown the manganese content of orchard grass (*Dactylis glomerata* L.), domestic rye (*Secale cereale* L.), tall oatgrass (*Arrhenatherum elatius* Beauv.), meadow fescue (*Festuca elatior* L.), timothy (*Phleum pratense* L.), Kentucky bluegrass (*Poa pratensis* L.), redtop (*Agrostis palustris* Huds.), bromegrass (*Bromus inermis*), and alfalfa (*Medicago sativa* L.). The samples used for analysis were collected from the experimental substation plots at Caldwell, Idaho. Just before the plots were grazed, samples from each were taken and sent to the laboratory for analysis. The samples were then dried, ashed, and analyzed as described in the following paragraphs.

METHODS OF ANALYSIS

Since the anhydrous sodium carbonate fusion of the ash in biological material had shown increased recovery of calcium and magnesium (3, 8), it was felt that the method might well be applied to the colorimetric determination of manganese. The simplicity of the method and its many advantages in manipulation recommended it as a rapid and accurate means of getting complete recovery of manganese in plant tissue.

One to two grams of the air-dry material to be analyzed was weighed into a platinum dish and ashed overnight in an electric muffle at 600° C. The ash was then fused with 3 g of anhydrous sodium carbonate. After cooling, the fused mass was placed in a 250-cc beaker and covered with a watch glass. Distilled water was added in sufficient quantity to moisten the sample and then 15 cc of 20-percent sulphuric acid (by volume) was added. When the fused material was dissolved, it was washed from the platinum crucible with

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a few cubic centimeters of 5-percent sulphuric acid. A few drops of 15-percent sodium bisulphite were added to the solution until all the manganese was reduced to manganous sulphate. The solution was then boiled to expel excess sulphur dioxide, filtered, and the acid-insoluble residue washed with several small portions of 5-percent sulphuric acid.

To the filtrate was added approximately 0.3 g of potassium periodate. The beaker was covered with a watch glass and its contents boiled for 5 minutes. The solution was allowed to stand for 1 hour at a temperature of 95° to 100° C. to insure complete oxidation of the manganese. The solution was then diluted to 90 to 95 cc with 5-percent sulphuric acid previously boiled with a little potassium periodate, and cooled to room temperature, the entire sample transferred to a 100-cc colorimetric tube and compared in a colorimeter with a standard manganese solution. A standard solution containing 0.0025 mg of manganese per cubic centimeter was a satisfactory one to use.

The chlorides were not removed from the samples; this step seemed unnecessary because of the small amount that is present in plant material. According to Willard and Greathouse (*14*), the presence of chlorides does not interfere with the final development of color, as the chlorides may be driven off by prolonging the time of oxidation and adding an excess of potassium periodate. Richards (*11*) has shown that excess acidity prevents full development of color or causes it to fade. The acidity, therefore, was kept between 5 and 6 percent. In the large number of samples analyzed no fading of color was observed. It was possible to let the samples stand for several hours and check the first reading, provided they were kept free from the fumes in the laboratory.

The marked agreement in the results of analyses of 1-g samples of the same grass (orchard grass), is shown in the following tabulation:

Sample no.—	Manganese (milligrams per kilogram)
1	177. 7
2	177. 7
3	176. 5
4	181. 7
5	178. 7
6	180. 0
7	179. 2
8	179. 2
Average	178. 8

As a further test of the method, known quantities of manganese were added to samples of air-dry timothy hay, and recovery determinations were then made. Table 1 shows that excellent recovery was obtained, considering the fact that a very small quantity of manganese was added as compared with that present in the sample. The difference in recovery of manganese was well within the experimental error of the colorimeter.

TABLE 1.—*Recovery of known quantities of manganese added as manganese sulphate to 2 grams of air-dry timothy hay*

Manganese added (milligram)	Total manganese found	Manganese in grass taken	Added manganese found	Recovery	Manganese added (milligram)	Total manganese found	Manganese in grass taken	Added manganese found	Recovery
	Milligram	*Milligram*	*Milligram*	*Percent*		*Milligram*	*Milligram*	*Milligram*	*Percent*
0.025	0.210	0.1825	0.0275	110	0.030	0.210	0.1825	0.0275	92
	.207	.1825	.0245	98		.212	.1825	.0295	98
	.206	.1825	.0235	94		.215	.1825	.0325	108
0.050	.232	.1825	.0495	99	0.020	.205	.1825	.0225	112
	.230	.1825	.0475	95		.200	.1825	.0175	87
	.235	.1825	.0525	105		.203	.1825	.0205	102
0.015	.197	.1825	.0145	97	0.035	.215	.1825	.0325	93
	.195	.1825	.0125	83		.218	.1825	.0355	101
	.195	.1825	.0125	83		.220	.1825	.0375	107

A comparison of the percentage recovery of manganese by the sodium carbonate fusion method, by the hydrofluoric-sulphuric acid digestion method (*1*), and by the official method (*5*) is presented in table 2. The hydrofluoric-sulphuric acid digestion method was used as a base of 100-percent recovery to facilitate the comparison. It will be observed that the results obtained by the anhydrous sodium carbonate fusion method showed very good agreement with those obtained by the hydrofluoric-sulphuric acid digestion method, but the recovery of manganese ranged from 15 to 35 percent lower by the official method than by the other two methods. The sodium carbonate fusion method has been used in a large number of analyses of plant material and has given very satisfactory results.

TABLE 2.—*Comparison of the 3 different methods used for the analysis of manganese in 7 grasses*

Grass	Hydrofluoric-sulfuric acid digestion of plant ash	Sodium carbonate fusion of plant ash	Official method	Sodium carbonate fusion / Hydrofluoric acid digestion	Official method / Hydrofluoric acid digestion
	Mg per kg	*Mg per kg*	*Mg per kg*	*Percent*	*Percent*
Orchard grass	150	150	122	100.0	81.3
Domestic rye	122	120	102	98.3	83.6
Tall oatgrass	110	111	94	100.9	85.4
Meadow fescue	105	107	90	101.9	85.7
Kentucky bluegrass	77	76	64	98.7	83.1
Bromegrass	150	152	110	101.3	73.3
Redtop	230	228	150	99.1	65.2

EXPERIMENTAL PLOTS

The soil used for the experimental plots is a Boise sandy loam. It is described (*4, p. 426*) as a

. . . . grayish-colored light sandy loam, with a soft, ashy feel, carrying a large amount of silt and having an average depth of about 2 feet. The subsoil of this type south of Boise River is a loam or clay loam which has an average depth of about 18 to 24 inches. This in turn is underlain usually with a sandy loam, but sometimes with sand, generally cemented together with calcium carbonate, forming a hardpan.

In the late summer of 1930 one area of a field was fenced and divided into 9 plots. These plots were about 25 feet wide and 20 rods long, the length of the plots being parallel to the slope of the field. All the plots had uniform drainage, slope, and irrigation. Prior to the experimental work the following crops had been grown on this soil: In 1921, corn; in 1922 and 1923, barley; in 1924 and 1925, wheat; and in 1926 to 1930, alfalfa.

The plots were grazed according to good pasture practice. Every 2 weeks during the grazing season dairy heifers were allowed to graze on the plots for 1 to 3 days. After the stock had been removed, the plots were irrigated, but no fertilizer was applied to any of them during the experiment.

Since the plots were grazed instead of clipped, definite yield data were not obtained. However, the approximate height of each variety of grass was determined at the time the sample was taken for analysis, that is, just before the heifers were admitted to the plots every 2 weeks throughout the grazing season. The fastest growing grass, tall oatgrass, averaged 6.4 inches in height; orchard grass, 3.6 inches; bromegrass, meadow fescue, redtop, timothy, and domestic rye, averaged 2.6 inches. Kentucky bluegrass, the slowest growing grass, averaged 1.9 inches. Alfalfa averaged 5.5 inches. All the grasses, and the alfalfa, grew more rapidly in the early part of the season.

A representative sample of soil from each of the plots was taken during the grazing season and analyzed for manganese and calcium carbonate, and the pH values were determined. The manganese was determined as described in the official methods (7). Calcium carbonate was determined by the Puri method (10) and the pH values by the hydrogen-electrode method. The soils of all the plots were uniform in manganese and calcium carbonate and in pH values (table 3).

TABLE 3.—*pH and percentage of manganese and calcium carbonate of the soil of 9 pasture plots*

Plot planted in—	Man-ganese	Cal-cium carbon-ate	pH	Plot planted in—	Man-ganese	Cal-cium carbon-ate	pH
	Percent	*Percent*			*Percent*	*Percent*	
Orchard grass	0.0706	1.00	7.86	Bromegrass	0.0715	0.75	7.90
Domestic rye	.0710	.75	7.23	Redtop	.0712	.75	7.86
Tall oatgrass	.0735	.75	7.85	Alfalfa	.0712	.75	7.72
Meadow fescue	.0703	.75	7.65				
Timothy	.0711	.75	7.55	Average	.07123	.777	7.72
Kentucky bluegrass	.0707	.75	7.86				

RESULTS OF ANALYSIS

Table 4 presents the analyses of samples of orchard grass, domestic rye, tall oatgrass, meadow fescue, timothy, Kentucky bluegrass, bromegrass, redtop, and alfalfa, taken throughout the grazing season.

TABLE 4.—*Manganese content (milligram per kilogram of grass on oven-dry basis) of 8 varieties of grasses and 1 alfalfa, grown under grazing conditions*

Date taken	Or-chard grass	Domes-tic rye	Tall oat-grass	Mead-ow fescue	Timo-thy	Ken-tucky blue-grass	Brome grass	Red-top	Alfalfa
1932									
June 6	268	197	105	174	86	69	172	188	61
June 20	240	130	81	127	86	77	172	161	41
July 4	292	145	91	160	97	70	166	161	39
July 18	240	120	90	149	97	75	172	214	49
Aug. 1	214	85	90	154	106	107	134	214	40
Aug. 15	170	97	82	184	150	83	134	161	47
Aug. 29	214	97	105	176	130	73	134	177	47
Sept. 11	158	90	121	154	130	75	107	161	47
Sept. 26	165	123	126	110	142	80	152	244	48
Oct. 11	114	135	113	124	120	72	203	240	47
Average	207.5	121.9	100.4	151.2	114.4	78.1	154.6	192.1	46.6

Wide variations are shown in the manganese content of samples of a single variety of grass taken on different dates. In spite of this, certain grasses showed a relatively higher manganese content than others. Orchard grass had the highest average manganese content of all the grasses. Next in order came redtop, bromegrass, meadow fescue, domestic rye, timothy, tall oatgrass, and Kentucky bluegrass.

Alfalfa had a lower manganese content than any of the grasses. Since comparative yield data were lacking, it was impossible to compare the total amount of manganese recovered by alfalfa with the total amount recovered by the grasses. A rapid-growing and high-yielding species like alfalfa, although low in manganese, would show a relatively high total recovery of manganese for the season.

Since the soils of the nine plots showed a uniform manganese content, and since all the plots had received the same treatment, it would seem that the difference in manganese content in the eight varieties of grasses was not due to the difference in manganese content of the soil or to a difference in soil conditions. Moreover, no correlation was found between the rate of growth of the eight grasses and their manganese content, nor between the rate of growth of a single variety of grass and its manganese content. It was concluded, therefore, that the difference in manganese content in the eight grasses was due to the difference in the capacity of the grasses for extracting manganese from the soil.

As a check on the foregoing conclusion the writer calculated, from the field data of Wiggans (*13*), the total amount of manganese recovered by seven of the grasses used in this experiment. Wiggans gives the average yield over a 4-year period of several grasses cut as pasture. His yield data, compiled by Ellenberger, Newlander, and Jones (*2, p. 6*), give the yield in pounds of dry matter per acre, as follows: Timothy, 1,454 pounds; redtop, 1,557 pounds; meadow fescue, 1,665 pounds; orchard grass, 1,711 pounds; Kentucky bluegrass, 1,387 pounds; bromegrass, 1,690 pounds; tall oatgrass, 2,044 pounds.

The total amount of manganese recovered for each grass in pounds per acre, based upon the preceding yield data, was as follows: Orchard grass, 0.0355 pound; redtop, 0.0299 pound; bromegrass, 0.0262 pound; meadow fescue, 0.0252 pound; tall oatgrass, 0.0205 pound; timothy, 0.0166 pound; and Kentucky blue grass, 0.0109 pound. With the

exception of tall oatgrass, there was a close correlation between the total amount of manganese recovered by the grasses during the grazing season and their manganese content. The exception of tall oatgrass was to be expected, as this was one of the fastest growing and largest yielding grasses.

Since complete information on the manganese requirements of animals, and on the effect of an excess or deficiency of manganese in grasses is not available, it is impossible to determine to what extent the feeding value of the pasture is affected by its manganese content. It is clearly shown from the data presented, however, that the manganese content of pasture may be increased by growing such grasses as orchard grass, redtop, bromegrass, and meadow fescue.

SUMMARY

A new method for the determination of manganese in plant material by the fusion of the plant ash with anhydrous sodium carbonate is presented. This method gives a greater recovery of manganese than is obtained by the official methods.

The manganese content of eight grasses is shown. The average manganese content (dry basis) ranged from 207.5 mg per kilogram for orchard grass to 78.1 mg per kilogram for Kentucky bluegrass. Alfalfa, with an average of 46.6 mg per kilogram was lower in manganese than any of the grasses.

The eight grasses varied markedly in their capacity to extract manganese from the soil.

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PLANT-TISSUE RELATIONS OF THE SUGAR-BEET CURLY-TOP VIRUS [1]

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INTRODUCTION

The distribution of virus in different organs of affected plants received attention from some of the pioneer investigators in the field of plant virus diseases. More recently, consideration has been given to the tissues that may be concerned in the increase and distribution of virus in the plant and to the production of primary and secondary pathologic symptoms. Enough evidence has been accumulated to indicate a wide range of variability among viruses in their relation to various tissues of affected plants.

The virus of true tobacco mosaic furnishes one of the best examples of rapid and extensive invasion of tissues. It seems to have a general systemic distribution in tobacco (*Nicotiana tabacum* L.) and probably invades nearly all the living cells of the plant. Certain other viruses have a more limited distribution and seem able to invade only specific tissues or parts. For example, it is doubtful whether the curl virus of raspberry (*Rubus trigosus* Michx.) occurs in tissue other than phloem, and the virus of the phony disease of peach (*Amygdalus persica* L.) is known to be restricted to the root system in the peach, though distinct pathologic symptoms occur on the tops of affected plants.

Considerable evidence, largely circumstantial, has been accumulated which indicates an intimate relationship between viruses and phloem tissue, and which may be summarized as follows: (1) In virus diseases, such as potato leaf roll and sugar-beet curly top, in which necrotic areas are characteristic, necrosis is largely restricted to the phloem where it begins; (2) in certain virus diseases, notably tobacco mosaic, sugar-beet curly top, and maize streak, the rate of virus spread in the plant is best explained by assuming that the phloem is the main channel of movement; (3) it is believed by some workers that most insect vectors habitually feed on vascular tissue, and this has been shown to be true in the case of the vector of sugarcane mosaic; (4) the virus of raspberry curl and that of two types of raspberry mosaic may be restricted in their movement through the plant by removing rings of bark. Furthermore, in curly top and certain other virus diseases the low percentage of infection obtained by artificial methods of inoculation may be due to inability, with the relatively crude technic available, to place the virus in susceptible vascular tissue without causing injury that inhibits development of the

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virus. Some of the evidence indicates the possibility of complete restriction of virus to the phloem tissue in a few diseases, although it cannot be contended that this may prove to have a general application.

The wide botanical range of plants affected by the curly-top virus offers possibilities for an extensive study of some of the relations of this virus to different plant tissues. Taking advantage of this fact, experiments have been performed with a view to securing data on such problems as that of determining the tissues in which the virus must be placed to produce infection, the tissues on which the vector of the virus of curly top feeds, the tissues from which the virus may be recovered, and the channels and rate of dispersion of the virus in the plant.

ARTIFICIAL INOCULATION

Infection of sugar beets (*Beta vulgaris* L.) or of other plants susceptible to curly top by other means than the feeding of leaf hoppers, *Eutettix tenellus* (Baker), has been obtained with difficulty and in only a very small percentage of the plants inoculated. Severin (*22*) [2] induced infection in beets by making repeated punctures with insect pins into the crown through drops of expressed beet juice. Carsner and Stahl (*8*) were successful in obtaining infection in only a few of a large number of plants inoculated by artificial means. Dana (*11*) in one experiment produced infection in 8 of 16 beet plants. In other trials in which beets, spinach (*Spinacia oleracea* L.), and tomato (*Lycopersicon esculentum* Mill.) were inoculated, a low percentage of infection was obtained.

An effective method of artificial transmission of curly top would facilitate materially the study of many of the problems presented by this disease and its causal agent. The results of artificial methods of inoculation, whether or not successful in producing infection, should throw some light on the general question of the plant tissues in which the virus may multiply and from which it can exert its effects on the plant as a whole. With these points in mind, a number of methods of inoculation were tried, most of which are already in general use.

The plants inoculated included sugar beet, Hubbard squash (*Cucurbita maxima* Duchesne), Turkish tobacco (*Nicotiana tabacum* L.), and Black Valentine bean (*Phaseolus vulgaris* L.). Affected specimens of all these plants and also macerated beet leaf hoppers were used as sources of inoculum. Plants of various ages and conditions of growth were used with the different methods of inoculation. The experiments were made at Riverside, Calif., from 1929 to 1932.

NEGATIVE RESULTS OF INOCULATIONS THROUGH XYLEM

In the earlier experiments attempts were made to infect through the xylem elements of the vascular bundles. Twenty plants having roots approximately 1 inch in diameter were taken from the soil, the lower third of the main root was cut away, and the cut surface of the remaining root was placed in centrifugalized juice from diseased beets. The plants were placed for 8 hours in a dry atmosphere, to increase transpiration, and were then transplanted to 6-inch pots. All remained healthy.

In a modification of the above experiment, 10 beets were transplanted to 3-inch pots, a portion of the main root of each beet being allowed to project through the drainage hole at the bottom of the pot. The 3-inch pots were set on the surface of soil in 6-inch pots with the projecting root embedded in the soil of the larger pot. After the plants had become adjusted to this new condition the smaller pot, with the projecting root system, was carefully removed from the 6-inch pot, and the exposed roots were washed free from soil and severely pruned. The 3-inch pots were then placed over containers so that the exposed part of the beet root was immersed in a liquid composed of 1 part juice from diseased beets and 3 parts tap water. The soil in the pots was allowed to become quite dry, and the plants were placed in a dry atmosphere to increase the amount of inoculum taken up by the root system. After 48 hours the plants were removed and transplanted to 6-inch pots. No disease developed in any of these plants.

In a later experiment, 20 rapidly growing beets having a crown diameter of about 1 inch were used. A hole was bored through the crown by means of a small-size cork borer. Glass tubing was inserted to a distance of about one fourth of an inch in each end of this hole. The beets were then joined in series by means of rubber tubing and connected to a liter flask containing centrifugalized juice from diseased beets. A layer of heavy oil was poured over the surface of the beet juice in the flask to reduce oxidation. A gravity flow of beet juice through the system was started, the juice being taken from near the bottom of the flask. The flow of beet juice was regulated to about 20 drops per minute by means of a pinchcock at the distal end of the system. This volume of flow was continued for 48 hours, with a change to fresh beet juice every 12 hours. None of these plants developed signs of curly top.

In the experiments described above it is reasonable to conclude that considerable beet juice was taken up by the tracheae of the inoculated plants and that this juice contained active virus. The failure to produce disease indicates that the curly-top virus does not pass from tracheae into cells or tissues that permit it to become established and to initiate pathologic symptoms.

RESULTS OF VARIOUS METHODS OF INOCULATION

Many plants have been inoculated by other methods. These methods consisted of puncturing leaves, cotyledons, and crowns of young plants through drops of inoculum by means of small needles; rubbing leaves with rolls of cheesecloth saturated with inoculum; and injecting inoculum into the hollow stems of squash and into the pith of tobacco by means of a hypodermic needle.

Inoculum was prepared in the following ways: (1) Diseased plants were ground in a meat chopper, the juice was expressed and centrifugalized, and the relatively clear liquid was decanted and used as inoculum; (2) viruliferous beet leaf hoppers were macerated in a small amount of water in a mortar and used as inoculum; (3) the surfaces of the crowns of diseased beets were cut away with a sharp knife and the exudate from the cut surface was collected and used as inoculum.

The results of these inoculations are given in table 1. Centrifugalized juice from diseased plants proved to be a very poor source of infectious material with the methods of inoculation employed.

Macerated leaf hoppers likewise were a poor source of infectious material, although the number of plants inoculated was too small to justify final conclusions. The best results were obtained from the use of exudate from the cut surface of diseased beets. With this material, 14 of the 124 plants inoculated became infected. Although this is a low percentage of infection, it is so much higher than that obtained by the use of expressed juice that it is worthy of further trial.

HOW THE BEET LEAF HOPPER FEEDS

With few exceptions, insects that are important vectors of plant viruses have mouth parts adapted for sucking plant juices. The feeding habits of a considerable number of species of sucking insects have been studied by several investigators who have determined the relation of feeding punctures to specific tissues. These investigations have dealt predominantly with insects not associated with the spread of plant viruses, though several of the species cause severe injury to their host plants as a result of the introduction of toxic substances.

TABLE 1.—*Results of artificial inoculation of sugar beet, Turkish tobacco, Hubbard squash, and Black Valentine bean with virus from different sources*

Inoculum	Plant inoculated	Method of inocula-tion [a]	Number of plants	
			Inoculated	Infected
Juice of beet	Beet	1	80	0
Do	Hubbard squash	1, 2	80	0
Do	do	4	20	0
Juice of Hubbard squash	Beet	2	80	0
Do	do	1	80	0
Juice of Turkish tobacco	Turkish tobacco	2	80	0
Do	do	1	20	1
Do	Beet	1	80	0
Juice of Black Valentine bean	Black Valentine bean	3	80	0
Crushed beet leaf hoppers	Beet	1	80	0
Phloem exudate from beet	do	1	124	14

[a] Numbers in this column refer to the following methods of inoculation: 1, Needle punctures into crown through drops of inoculum; 2, needle punctures into crown through diseased leaves; 3, gentle rubbing of leaves with a roll of cheesecloth saturated with inoculum; 4, inoculum injected into the hollow stem of squash or into the pith of tobacco by means of a hypodermic needle.

Büsgen (*6*), Davidson (*12*), Horsfall (*16*), Kenneth M. Smith (*25*), and others have shown that aphids, which constitute by far the most important group of vectors of plant viruses, feed on the phloem tissues of the plants on which they live. Other sucking insects feed on parenchyma or vascular tissue or both, depending on the species. Leaf hoppers as a group, obtain food material from a number of tissues. Smith and Poos (*24*) found that of 6 species studied 5 fed primarily on the mesophyll of the leaf and 1 on the phloem.

In only a few instances have careful studies been made of the feeding habits of insects in relation to transmission of plant viruses. Brandes (*5*) has shown that *Aphis maidis* Fitch, a vector of sugarcane mosaic, makes the phloem its primary objective. The stylets of this insect penetrate the epidermis directly, pass through cells and intercellular spaces of the underlying tissues, and terminate in the phloem of a vascular bundle. Kenneth M. Smith (*26*) states that the species of aphids that transmit potato leaf roll, as well as species that do not transmit this disease, feed on the phloem.

No information has been published regarding the tissues from which the beet leaf hopper obtains its food supply. Observations on leaf hoppers caged on beets indicate that they prefer the veins. This preference is especially noticeable if the leaf hoppers are feeding on petioles. In the sugar-beet petiole there are normally five or more large veins and several smaller ones arranged in an arc beneath the convex surface. The larger veins are deep-seated. The smaller veins vary in this respect, but those in the acute angles of the petioles are always very close to the surface. In feeding, leaf hoppers arrange themselves in greatest numbers along these angles as if seeking the smaller and more superficial veins.

To supplement these observations on the feeding of the beet leaf hopper, more detailed investigations have been undertaken. This work has included a microscopic study of mouth parts inserted in the tissue, and similar studies of the feeding punctures in the beet petiole by means of freehand sections of fresh petioles and by means of embedded and stained material.

In obtaining mouth parts fixed in feeding position, leaf hoppers after being starved overnight were placed on beet petioles and allowed to feed until quiet. They were then subjected to a temperature of about 28° F. for several minutes or until they became inactive. A capillary pipette filled with ether was applied to the posterior end of the abdomen of each insect that remained undisturbed on the petiole. The etherized insects were covered with melted agar to fix them firmly in place. Portions of petiole with the agar-embedded leaf hoppers were killed and fixed in Schaffner's chromo-acetic solution, and sectioned in the usual way. A very slight movement of the leaf hoppers before or during the killing and fixing processes resulted in partial or complete withdrawal of the stylets. Many leaf hoppers were found with stylets exserted from the labium but not inserted in the tissue, or only partly so. However, several leaf hoppers were satisfactorily fixed with the mouth parts apparently in normal relation to the plant tissue.

In further studies leaf hoppers were allowed to feed from 12 to 24 hours on petioles and were then removed. Some of the petioles were sectioned immediately; others were killed, embedded, sectioned, and stained. The line of puncture is quite evident in fresh material as well as in stained sections. In penetrating the tissues the leaf hoppers form a sheath which completely encases the stylets. After the stylets are withdrawn this sheath remains and a definite line through the center marks the position of the stylets. In live petioles the sheath when first laid down is almost colorless but soon takes on a yellowish coloration which clearly differentiates it from the plant tissue. In prepared sections it takes a deep safranine stain with the safranine-Delafield's haematoxylin combination (fig. 1).

A study of leaf-hopper mouth parts in feeding position in conjunction with a study of numerous punctures in fresh and prepared material furnishes a complete picture of the relation of mouth parts to the various tissues of the plant during feeding. These studies have shown clearly that the leaf hopper is able to penetrate cell walls without difficulty (figs. 2 and 3). The line of puncture extends from the epidermis through and between cell walls of the subepidermal layers, frequently to vascular bundles. The path of penetration usually is

straight and directed toward a vein. However, the path may be curved and frequently is branched near the tip (fig. 1, *A*). Apparently the stylets can be bent in only one direction at a time, but by partly withdrawing them and inserting them in another direction the leaf hopper is able to explore a considerable area. Some trails curve toward a vascular bundle from an initial direction that would have
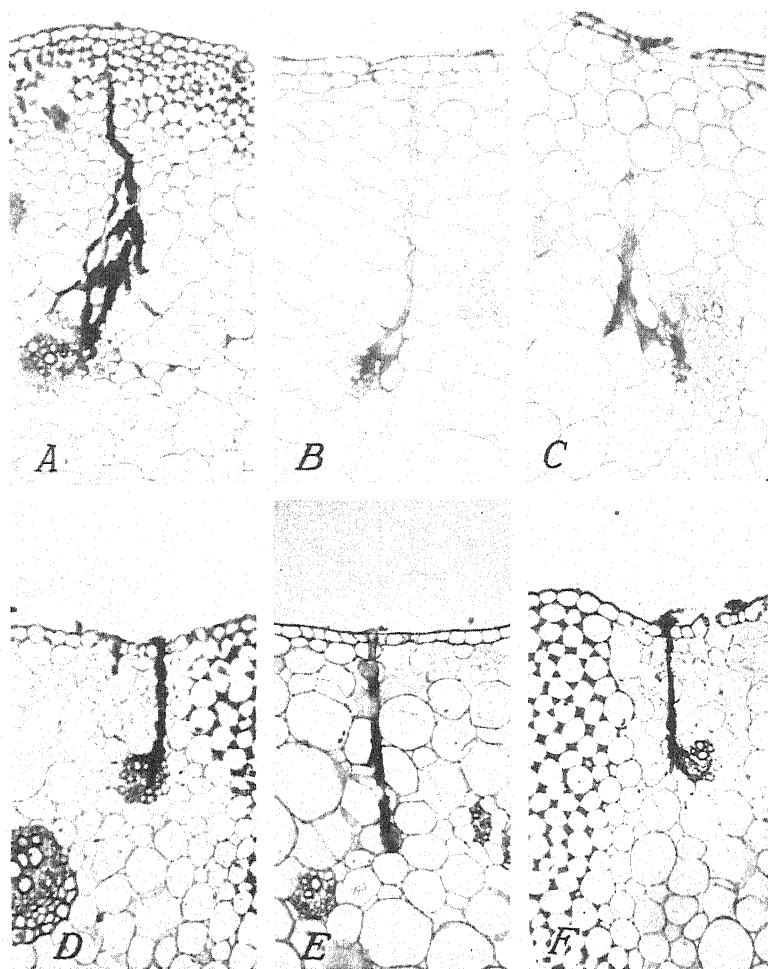
FIGURE 1, *A–F.*—Path of feeding punctures of *Eutettix tenellus* in beet petioles. Results of probing are shown in *A* and change of direction to reach vascular bundles in *B* and *C*. A puncture terminating in parenchyma is shown in *E*. × 90.

terminated in parenchyma (fig. 1, *C*). Punctures made from the xylem side of the petiole usually veer away from the middle of the bundle and enter the phloem from one side. A few instances of xylem invasion were noted in which copious quantities of exudate were deposited in the tracheae (fig. 3). Whether this happened by chance or whether leaf hoppers extract water or food from the xylem is difficult to determine. However, the number of punctures terminating in the

phloem and the amount of probing sometimes done, apparently in order to locate the phloem, indicate that this is the tissue of primary importance in supplying food.

One hundred punctures were counted and classified on the basis of the tissue in which they terminated. Of these, 24 terminated in or near the phloem of small veins; 22 terminated in or near the phloem of large veins; 46 terminated in parenchyma outside of the bundles but began from points from which bundles could have been reached.
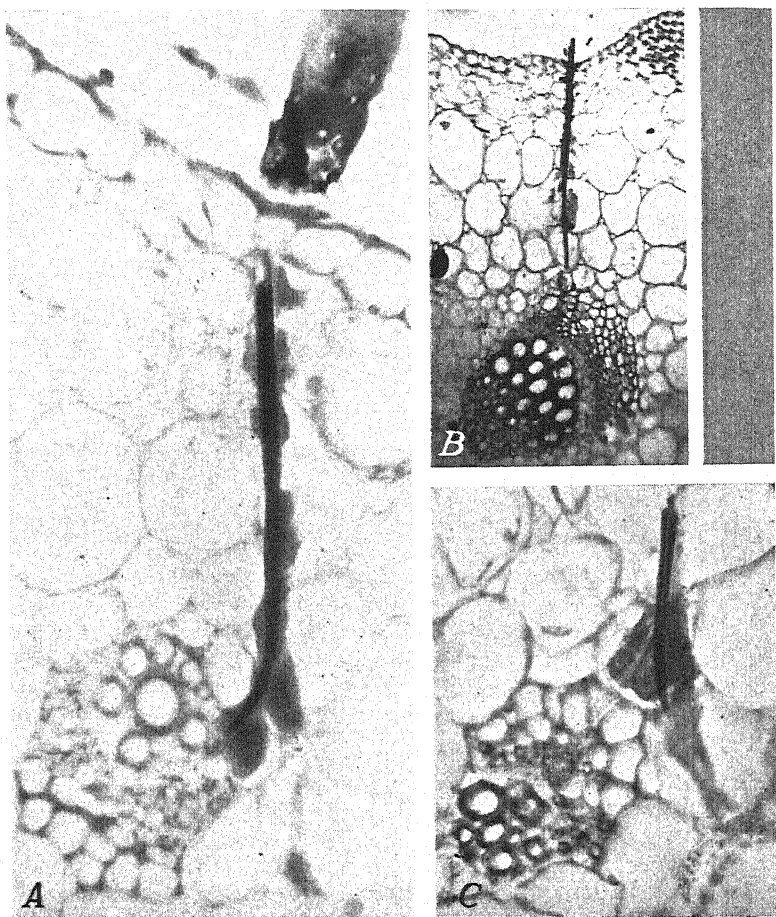
FIGURE 2.—*A-C*, Cross sections of beet petioles. The stylets of *Eutettix tenellus* are embedded in the tissue in the normal feeding position. × 90.

Only 8 trails were found in the parenchyma of the concave side of the petioles originating from points from which bundles could not have been reached. It should be stated that'.the section from which these counts were made came from small petioles on which large numbers of leaf hoppers had fed. Larger petioles and smaller numbers of leaf hoppers might be expected to give different results and probably would reduce the number of punctures terminating in the parenchyma outside of the bundles, many of which were very shallow and were

probably made by leaf hoppers disturbed before maximum penetration had been effected. None of the punctures in the parenchyma was branched nor was there other evidence that the leaf hopper spent much time in exploring such areas. In view of these facts and of evidence to be presented later showing that the leaf hopper derives very little of the life-sustaining materials from parenchyma, it seems probable that in making these punctures the leaf hoppers were merely searching for a more desirable medium from which to extract food.

Several investigators have mentioned the sheath material found in the feeding punctures of sucking insects, but there is a lack of agreement as to whether the sheath material is of plant or of insect origin.

Büsgen (*6*) is of the opinion that the sheath laid down by the insects that he studied was composed of material excreted by the insect. Davidson (*12*) considers that the sheath wall of *Aphis rumicis* L. is "composed of substances produced by the reaction of the saliva on the cell sap." Horsfall (*16*) found that the sheath in the feeding punctures left by certain aphids contains proteid material and calcium pectate, and suggests that it is laid down by the plant cells in response to a wound stimulus though the proteid material may possibly be injected by the insect. King and Cook (*17*) suggest that the sheath produced by the sucking insects that they studied results from the action of insect saliva on the middle lamella. F. F. Smith (*23*) has shown that the sheath material in the punctures produced by the potato leaf hopper and the three-cornered alfalfa hopper

FIGURE 3.—Stylets of *Eutettix tenellus*, showing their relation to the tissues of the beet petiole during feeding. (Drawing made with the aid of a camera lucida.) × 180.

is largely of insect origin and contains no plant substances with the possible exception of pectose. Brandes (*5*) states that the sheath laid down by *A. maidis* is composed of material given off by the insect. It is obvious that the sheath material formed by *Eutettix tenellus* is composed of material that is given off by the leaf hopper itself. This was demonstrated by a method similar to that described by Fife (*14*), which involved mounting a live leaf hopper under a microscope in such a position as to have the mouth parts inserted horizontally through a membrane into a liquid in the field of vision. In this position the insect may be watched in the process of forming a sheath. Many individuals begin the discharge of a colorless material as soon as the mouth parts penetrate the membrane and continue as the stylets are inserted farther into the medium. The discharge coagulates almost

immediately and forms a distinctly visible hyaline sheath around the stylets, which in thickness and general physical properties is similar to the sheath found in freehand and stained sections of the beet petiole. Withdrawal of the stylets leaves a very definite line marking their position. With repeated penetration and partial withdrawal of the stylets a considerable mass of exudate is built up in which stylet trails extending in many directions are visible.

The materials deposited in the plant tissues by nonviruliferous leaf hoppers evidently cause very little injury to the plant as a whole, since a beet plant of average size will support a large leaf-hopper population for a considerable period with no marked ill effect.

Further investigations were made to determine the reaction of individual cells in different types of beet tissue to feeding punctures of nonviruliferous leaf hoppers. Large numbers of leaf hoppers were fed on beet petioles 24 hours and then removed. Microscopic examinations of freehand sections of these petioles were made daily for 10 days and at 5-day intervals thereafter, the last examination being made 20 days after feeding. The sheaths were at first hyaline but soon became yellowish or yellowish brown. They maintained their original relations to the cells to a remarkable degree. By the tenth day, in some instances, the sheath material was displaced in some of the cells and had shrunken slightly. It was still present and easily traced, however, on the twentieth day.

Where collenchyma was traversed, the yellowish color of the sheath was imparted to the thickened parts of the cell walls. This was true also of the cell walls of the bundle cap. Other cells in the path of punctures did not show this change in color of walls. The large parenchyma cells through which the sheaths passed reacted in different ways but all retained their turgidity for 20 days after the punctures were made. Some remained apparently normal, even retaining normal-appearing chloroplasts along with sheath material. Others had a distinctly granular protoplasmic structure and the nucleus in some cases was granular and irregular in outline. The cells were apparently very rarely dead even after 20 days. The cell-wall discoloration in the collenchyma and bundle cap had almost completely disappeared after 15 days, and the vascular bundles seemed normal except for the sheath material remaining in the cells.

Assuming that the phloem is at least the chief reservoir of virus and the place of most rapid multiplication, as stated previously by Brandes (5) in the case of *Aphis maidis*, it would be difficult to imagine a mechanism more perfectly designed for virus extraction and introduction than that possessed by the beet leaf hopper. The laying down of a sheath around the mouth parts as they penetrate probably effectually seals off all contents of cells external to the phloem. The introduction of sheath material into the phloem insures the introduction of salivary secretions into this tissue and probably accounts for the introduction of virus. This virus is liberated into a medium rich in nutrients and in a tissue physically adapted for the rapid distribution of inoculum to various parts of the plant, especially to the rapidly growing areas. The same insect mechanism is equally efficient in removing virus directly from the phloem without having the virus come in contact with cell contents of parenchyma tissue or with external agents.

TISSUES FROM WHICH THE LEAF HOPPER OBTAINS FOOD AND EXTRACTS VIRUS

Attempts were made to segregate certain types of beet tissue and to determine their virus content by testing the ability of leaf hoppers to obtain virus from them. These experiments have consisted chiefly of segregating parenchymatous tissues in different parts of the plant and comparing their virus content with adjacent tissues containing vascular bundles. Tissue in which there are no vacsular elements may be isolated from the ventral side of large petioles, from the crown of large beets, from the pith of the flowering stalk, and from seeds in an early stage of development.

However, before accurate conclusions regarding the virus content of tissues from these various sources may be drawn from the results of leaf-hopper transmission experiments, it is necessary to know the effect of the tissues on the leaf hopper and the relative amounts of materials that the leaf hopper is able to extract from them. To throw some light on this problem, the mortality of the leaf hoppers having access to parenchyma tissue from each of the sources just mentioned has been compared with the mortality of leaf hoppers having access to adjacent tissue containing vascular elements. The average length of life of leaf hoppers on the different tissues is taken as a rough relative measure of the food and other materials obtained.

FIGURE 4.—Mortality of *Eutettix tenellus* on different types of beet tissue at 65° to 75° F.

LEAF-HOPPER MORTALITY ON DIFFERENT TYPES OF BEET TISSUE

Mortality tests were made in two separate experiments. In the first experiment, mortality of leaf hoppers having access to vascular tissue of petioles was compared with that of an equal number of leaf hoppers having access to parenchyma of the ventral side of petioles. The experiment was started with 10 petioles for each type of feeding, and 10 leaf hoppers were placed on each petiole. Fresh petioles were supplied every 48 hours. The petioles were covered with a thick coating of paraffin, with strips of paraffin about one fourth of an inch wide and 3 inches long removed to expose parenchyma tissue on the concave side in one series and vascular tissue along the acute angles of the petioles in another series. As a further check on these treatments, a third lot of 100 leaf hoppers was placed in small cages where they had access to tap water through a parchment membrane, and a fourth lot of 100 was placed in small cages without food or water. The experiment was run at relatively low temperatures (60°–75° F.) and discontinued at the end of 10 days. The results are shown graphically in figure 4.

All leaf hoppers receiving neither food nor water were dead at the end of the second day. The mortality curve of the lot receiving parenchyma is roughly parallel with that of the lot receiving tap water, although the death rate is slightly lower in the latter group; mortality reached 100 percent on the seventh and ninth days, respectively. The leaf hoppers having access to the vascular tissue thrived much better, only 39 percent being dead at the end of the tenth day.

In a second experiment, tissue from additional sources was used and the leaf hoppers were kept at a temperature of 90° to 100° F. The different lots of insects in materials available for this experiment were given, respectively, (1) neither food nor water, (2) tap water, (3) parenchyma of the petiole, (4) vascular tissue of the petiole, (5) young seeds, (6) hull of the seed ball, (7) pith from the crown and flowering stem, and (8) tissue containing vascular elements, from areas adjacent to the pith of the flowering stalk and the crown. The experiment was run in duplicate series, 50 leaf hoppers being used in each treatment in each series. The test was discontinued at the end of 48 hours. The results are shown graphically in figure 5.

As measured by the control treatment in which the leaf hoppers received neither food nor water, each of the types of tissue on which the insects were allowed to feed yielded some life-sustaining materials. Young seeds proved to be poorest in this respect, probably in part be-

FIGURE 5.—Mortality of *Eutettix tenellus* on different types of beet tissue at 90° to 100° F.

cause of their tendency to dry very rapidly. The mortality curve, however, is steep for parenchyma from all sources except the flowering stalk and crown.

In general, these experiments indicate that parenchyma is unfavorable for the beet leaf hopper. Parenchyma of the petiole seems to be little better than tap water. Parenchyma of the flowering stalk and crown is more favorable and ranks in food value next to tissue containing vascular elements. It is worthy of note that parenchyma from the flowering stalk and crown is higher in sugar than parenchyma from other sources, and it is possible that this greater sugar content is responsible for the prolonged life of the leaf hoppers. Normally, of these types of parenchyma, only that from the petiole is available to leaf hoppers. These experiments furnish additional evidence to support the view that the beet leaf hopper is chiefly dependent on the phloem for its food.

The next question of importance is whether the leaf hopper feeds sufficiently on parenchyma from the various sources to acquire virus if it is present. Experiments have indicated that a relatively short period of feeding is sufficient for leaf hoppers to acquire virus from

diseased beet leaves. A few individuals have become viruliferous after a 1-minute feeding period, and larger numbers acquire virus as the feeding period is increased. Of 150 leaf hoppers fed singly on diseased beet leaves, 11 percent acquired the virus in 5 minutes. In another test with the same number of leaf hoppers, 23 percent became viruliferous after a 10-minute feeding. Since the evidence shows that the beet leaf hopper acquires enough material from parenchyma to appreciably prolong its life, and since the leaf hopper acquires the virus in a very short period of feeding on diseased beet leaves, it seems reasonable to assume that the leaf hopper may be used to furnish evidence regarding the presence or absence of virus in parenchymatous tissue despite the fact that parenchyma is not a very favorable source of food.

VIRUS CONTENT OF TISSUES OF BEET PLANT

Assuming that the method just described affords a means of testing for the presence of virus, nonviruliferous leaf hoppers have been given access to various types of tissue isolated from diseased beets. At the time the leaf hoppers were given access to parenchyma a second group of leaf hoppers was placed in an empty cage to serve as a check on the feeding of the leaf hoppers on parenchyma. When all the leaf hoppers serving as checks were dead the insects surviving on parenchyma were transferred to seedling plants. These checks were used in all tests except those involving the parenchyma of the petiole.

PARENCHYMA OF PETIOLE

Large petioles from beets infected during the current season were covered with paraffin, and strips of this were removed to expose parenchyma or vascular tissue, as described previously. Forty non-viruliferous leaf hoppers were allowed to feed on each petiole, 20 for 24 hours on parenchyma and 20 for 24 hours on vascular tissue. One half of the petioles had parenchyma exposed during the first 24 hours and vascular elements exposed during the second 24 hours, and the other half had the exposures made in reverse order. At the end of the feeding period the leaf hoppers were divided into lots of 5 each and caged on healthy plants. In this manner 8 healthy sugar-beet plants were inoculated from each petiole; 4 plants by means of leaf hoppers which had access to parenchyma and 4 by means of leaf hoppers which had access to vascular elements. One hundred and four plants were inoculated by means of leaf hoppers from each type of tissue. Of the 104 plants inoculated by means of leaf hoppers from vascular tissue, 42 became infected; whereas, of the 104 plants inoculated by means of leaf hoppers from parenchyma, only 1 became infected. The results of this experiment are shown in table 2.

In a second experiment, petioles were taken from plants that had been diseased several months and on which petioles had been produced subsequent to infection. The plan of this experiment was the same as that just described, except that larger numbers of petioles were used and only one lot of leaf hoppers was given access to each petiole. In this test, of the 80 plants inoculated by means of leaf hoppers from tissue containing vascular elements, 37 became infected; whereas, of the 80 plants inoculated by means of leaf hoppers from parenchyma tissue, none showed any sign of disease (table 2).

TABLE 2.—*Virus content of sugar-beet tissues as indicated by leaf-hopper tests*

Tissue tested	Leaf hoppers fed	Plants inoculated [a]	Plants infected	
	Number	*Number*	*Number*	*Per cent*
Vascular tissue, petiole of first-year beets	520	104	42	40.3
Parenchyma tissue, petiole of first-year beets	520	104	1	.9
Vascular tissue, petiole of second-year beets	400	80	37	46.2
Parenchyma tissue, petiole of second-year beets	400	80	0	.0
Vascular tissue, flowering stalk	420	84	62	73.8
Pith of flowering stalk	420	84	0	.0
Vascular tissue below crown	600	120	58	48.3
Parenchyma tissue below crown	600	120	9	7.5
Outer hull of seed ball	200	40	11	27.5
Young seeds	200	40	0	.0

[a] 5 leaf hoppers were placed on each plant inoculated.

PARENCHYMA OF CROWN

Tissue selected as containing no vascular elements was taken from the crown of large diseased beets and placed in a cage containing non-viruliferous leaf hoppers that had been starved overnight. A second lot of tissue containing vascular elements was taken from the portion of the beet adjacent to the first selection and exposed to a second lot of leaf hoppers. After the leaf hoppers had been allowed a feeding period of 5 hours they were divided into lots of 5 each and placed on healthy beet seedlings. One hundred and twenty plants were inoculated by means of leaf hoppers from each of the two food sources. In this experiment, of the 120 plants inoculated by means of leaf hoppers from tissue containing vascular elements, 58 became infected; and of the 120 plants inoculated by means of leaf hoppers from tissue containing no vascular elements, 9 became infected.

In the foregoing experiment, tissue was taken from six beets and the tissue from each beet was used as a separate test. For the six beets the number of infections resulting from the leaf hoppers that had access to parenchyma tissue was, respectively, 0, 1, 5, 0, 3, and 0. The infections resulting from leaf hoppers that had fed on adjacent vascular tissue from the same beets were, respectively, 6, 9, 18, 16, 9, and 5. Each value represents the number of infections obtained from inoculating 20 plants.

These results seem to demonstrate that virus does occur in some types of parenchyma tissue. Perhaps if virus occurs in any parenchyma tissue of the plant it would be expected to be present in the parenchyma that lies immediately below the growing point and closest to the actively growing areas of the beet crown. However, even there it seems to occur in diminished concentrations—in some cases in concentrations too low for relatively large numbers of leaf hoppers to pick it up.

PITH OF FLOWERING STALK

Large fruiting stalks were selected from plants that had been infected the season prior to flowering. Portions of pith containing no vascular tissue were removed and exposed to the feeding of non-viruliferous leaf hoppers. A second group of nonviruliferous leaf hoppers was allowed to feed on tissue containing vascular elements selected from the area immediately adjacent to the pith that was used as food for the first lot. Eighty-four plants were inoculated from each lot of leaf hoppers. Of the 84 plants inoculated by means of the leaf

hoppers from tissue containing vascular elements, 62 became infected; whereas of the 84 plants inoculated by means of the leaf hoppers from pith, none showed any sign of disease.

YOUNG SEEDS

Severin (*21*) has shown that there is no seed transmission of the curly-top virus in beet. Since this is true, the question arises as to whether the virus never gains access to the seed in any stage of its development or whether it may be present in certain early stages of seed development and become inactivated as the seed matures.

To obtain information as to whether virus occurs in seeds in the earlier stages of their development, young seeds from diseased plants were separated from the surrounding tissue and placed in cages where nonviruliferous leaf hoppers had access to them. Very young seeds having a high water content were selected. As a check on the virus content of the nearby tissue, the hulls of the seed balls from which the seeds were removed were placed in cages with other nonviruliferous leaf hoppers. After periods of several hours the leaf hoppers were divided into groups of five each and placed on seedling beets. Of the 40 plants inoculated by means of leaf hoppers from hulls, 11 showed signs of the disease; whereas of the 40 plants inoculated by means of leaf hoppers from seeds, none became infected.

Since no virus was obtained from the seeds by the leaf hoppers, it seems probable that seeds contain no virus even in the early stages of their development and that virus may not be able to pass from the plant into the seed. Therefore, absence of seed transmission may be due to a barrier between the embryo and the mother plant which, although permitting passage of water, mineral elements, and elaborated foods, restrains or inactivates the virus.

CONCENTRATION OF VIRUS IN PHLOEM EXUDATE

The foregoing experiments show that leaf hoppers readily acquire virus from vascular tissue and rarely obtain it from other tissues. Since the xylem elements evidently do not carry any considerable amount of virus, the phloem must contain at least the greater part of the virus in the vascular elements. Liquid from phloem tissue may be obtained by making cuts across the tops of beet roots. In a few minutes drops of exudate appear above the severed ends of vascular bundles and may be collected with capillary tubes and used in artificial feeding tests with leaf hoppers. Attempts were made to compare the relative virus concentration of such exudate with that of the expressed juice from the entire beet. In these tests, drops of exudate from diseased beets were placed on a parchment membrane. Nonviruliferous leaf hoppers were allowed to feed on the exudate through the membrane for about 4 hours and then were caged singly on seedling beets.

A second lot of nonviruliferous leaf hoppers were allowed to feed on expressed beet juice and then were caged singly on seedling beets. The results shown in table 3 indicate that more virus is available to the leaf hopper from phloem exudate than is available from expressed juice from the entire beet. Of the 104 leaf hoppers fed on phloem exudate, 33 produced infection; whereas of the 104 leaf hoppers having access to expressed beet juice, only 4 gave evidence of having acquired virus.

TABLE 3.—*Virus content of phloem exudate and expressed beet juice as indicated by leaf-hopper tests*

Material tested	Plants in-oculated [a]	Plants infected	
	Number	*Number*	*Percent*
Exudate from phloem of beet root..	104	33	31.7
Expressed juice from beet root..	104	4	3.8
Exudate from beet petioles...	24	7	29.1
Expressed juice from beet petioles...	24	0	.0

[a] 1 leaf hopper was placed on each plant inoculated.

It is noted frequently that drops of exudate collect on the leaves and petioles of rapidly growing beets that have been recently infected. This exudate has long been considered to come from the phloem. Recently, Esau (*13*) has made histological studies of diseased beets and described the path which this exudate takes in moving from the phloem to the exterior. By means of exudate of this type further feeding tests were made to determine virus concentration. These tests were carried out as already described, 24 leaf hoppers being given access to exudate and an equal number having access to expressed juice from beet petioles. In this test, 7 leaf hoppers acquired the virus from exudate, whereas none was found to be viruliferous after feeding on expressed juice.

In connection with the experiments that indicate a very low concentration of virus in types of tissue other than phloem, these tests with phloem exudate seem to demonstrate conclusively that the chief virus reservoir in the sugar beet is phloem tissue. Phloem exudate with its high virus content may prove valuable in virus purification experiments and in work dealing with properties of the virus.

MOVEMENT OF VIRUS IN DIFFERENT TISSUES
GRAFT UNIONS OF SUGAR BEET

Twenty healthy sugar-beet plants having main roots approximately three fourths of an inch in diameter were taken from the soil, and a portion of one side of each root was removed by means of a sharp knife. Twenty diseased beet plants were treated in a similar manner. The cut surface of each diseased beet was placed in contact with the cut surface of a healthy beet and the two plants firmly bound together and potted. Symptoms of curly top began to appear on the new leaves of the inoculated beets in 3 weeks. Of the 20 healthy beets, 17 became diseased. In the case of the 3 beets that did not develop symptoms, the diseased member of the pair died probably before union was complete.

GRAFT UNIONS OF TOBACCO

Experiments were conducted with Turkish tobacco to determine at what stage in the development of a graft union the curly-top virus passes from a diseased scion to a healthy stock. Healthy tobacco plants were cut back to a height of about 8 inches, and 3 inches of stem from a diseased plant was grafted to each healthy plant. Cuts were made at angles that afforded considerable surface contact. Grafts were removed from stocks at 24-hour intervals for 15 days and the results on the inoculated plants noted (table 4).

TABLE 4.—*Time required for infection of healthy tobacco stocks by scions from diseased plants*

Period between grafting and removal of diseased scion (days)	Plants grafted	Plants infected		Period between grafting and removal of diseased scion (days)	Plants grafted	Plants infected	
	Number	*Number*	*Percent*		*Number*	*Number*	*Percent*
1	10	0	0	9	12	7	58
2	10	0	0	10	10	7	70
3	10	0	0	11	10	8	80
4	10	0	0	12	10	10	100
5	10	0	0	13	10	9	90
6	13	0	0	14	10	10	100
7	11	3	27	15	10	9	90
8	12	5	41				

No infection occurred until the seventh day. The number of infected plants increased from 27 percent on the seventh day to 100 percent on the twelfth day. The union between the stock and the scion was examined for all the time intervals used. At the end of 3 days a definite union was found which was complete enough to make necessary the use of an appreciable amount of force in removing the scion. Graft unions of different ages from 3 to 9 days were killed, embedded, and sectioned, and then were examined under a compound microscope. In all the specimens sectioned, the union at the end of the third day was composed wholly of meristematic tissue. This rapidly differentiated into other tissues and in the 5- and 6-day-old unions the beginnings of tracheal elements were clearly evident. In the 7-, 8-, and 9-day-old unions, apparently mature tracheal elements with pits and rings were distinctly visible. The walls of these elements were lignified, as indicated by their taking a deep safranine stain. Phloem elements could not be clearly identified, but strands of elongated cells paralleling the tracheae were observed, which may well have included functional phloem.

The foregoing experiment seems to demonstrate that in tobacco infection does not result from contact of cut surfaces and that virus does not move through newly formed meristem in a period of from 2 to 4 days. Infection apparently does not occur until after tracheal elements, and probably phloem elements, connect the stock with the scion. Since the virus seems unable to pass out of tracheae into other cells and become established sufficiently to produce disease, and since it also seems unable to pass through meristem or young parenchyma, it appears extremely probable that in tobacco grafts the virus moves in the phloem in crossing a graft union.

REMOVAL OF RINGS OF BARK [3] AND INTERNAL PHLOEM

Killing portions of stems and removing rings of bark have been the methods used by several investigators to obtain evidence regarding the tissues in which virus is dispersed through plants.

The writer (*2, 3*) found that the virus causing curl of raspberry and two viruses causing mosaic of raspberry may be limited in their movement through the plant by removing rings of bark.

In tomato, according to Caldwell (*7*), the virus of mosaic bridged an area on the stem from which a ring of bark was removed. As

[3] The term "bark" as used in this paper signifies all tissue of the stem from epidermis to cambium, inclusive.

Caldwell points out, however, the tomato has a weak development of internal phloem, so that it is not clear whether the virus passed through the woody cylinder, the pith, or the internal phloem. The same investigator conducted experiments to determine whether the virus moved through dead portions of stem. Of the 26 plants having portions of the stems treated with chloroform, the virus in 14 did not cross the treated area. In 12 plants, virus was present in noninoculated parts. These exceptions are attributed to incomplete killing of the stem, regeneration of tissue, and accidental infection. In plants having portions of the stems killed by steaming, the virus was held in the inoculated parts for several weeks in 20 of the 21 plants used. The one exception was thought to be due to accidental infection and not to movement of virus across the steamed area.

The virus causing curly top has certain characteristics that render it convenient for experimental use in tests involving tissues through which movement occurs. One of the most important of these is its failure to produce infection except as inoculated into plants by its specific vector, thus reducing to a minimum the chance infection of noninoculated parts of plants kept for long periods of time. Moreover, the virus causes disease in a large number of plant species, thus making available a wide range of anatomical types for experimental use.

Since the sugar beet cannot be employed in ringing experiments because of its anatomical structure, it was necessary to choose some other susceptible plant for such experiments. In making a survey of susceptible plants of a more or less woody nature, tests were made on two species of tobacco, namely, common tobacco (*Nicotiana tabacum* L.), Turkish variety, and tree tobacco (*N. glauca* Graham). These plants grow well under a wide range of greenhouse conditions, are easily propagated at all seasons from seeds or cuttings, and are very satisfactory for experiments involving grafting. Moreover, the presence of an internal phloem makes these species suitable for experiments on the movement of virus in the phloem not possible in most other types of woody plants. Each of these species has been used in a fairly extensive series of ringing experiments.

NICOTIANA TABACUM

Turkish tobacco has only a medium degree of susceptibility to infection by the curly-top virus, but symptoms of disease are characteristic and well marked. Infection may be induced by leaf hoppers or by grafting. In most of the experiments about to be described leaf hoppers were used in making inoculations.

The first experiments with Turkish tobacco were planned to determine whether the virus would pass downward through a killed portion of stem. Plants approximately 2 feet tall were cut back to a height of about 10 inches and the buds in the leaf axils allowed to grow to a length of 1 to 2 inches. A portion of the stem below the second or third bud from the top was incased in a celluloid cylinder, the bottom being closed around the stem. Hot paraffin was poured into the cylinder. This killed the tissues and protected and supported the killed area. After a portion of the stem had been killed in this manner, leaf hoppers were allowed to feed on the top bud. Distinct

symptoms of curly top appeared in an average period of 7 days on all the inoculated buds of the 10 plants used. The inoculated parts lived for an average period of 16 days. All plants were held for
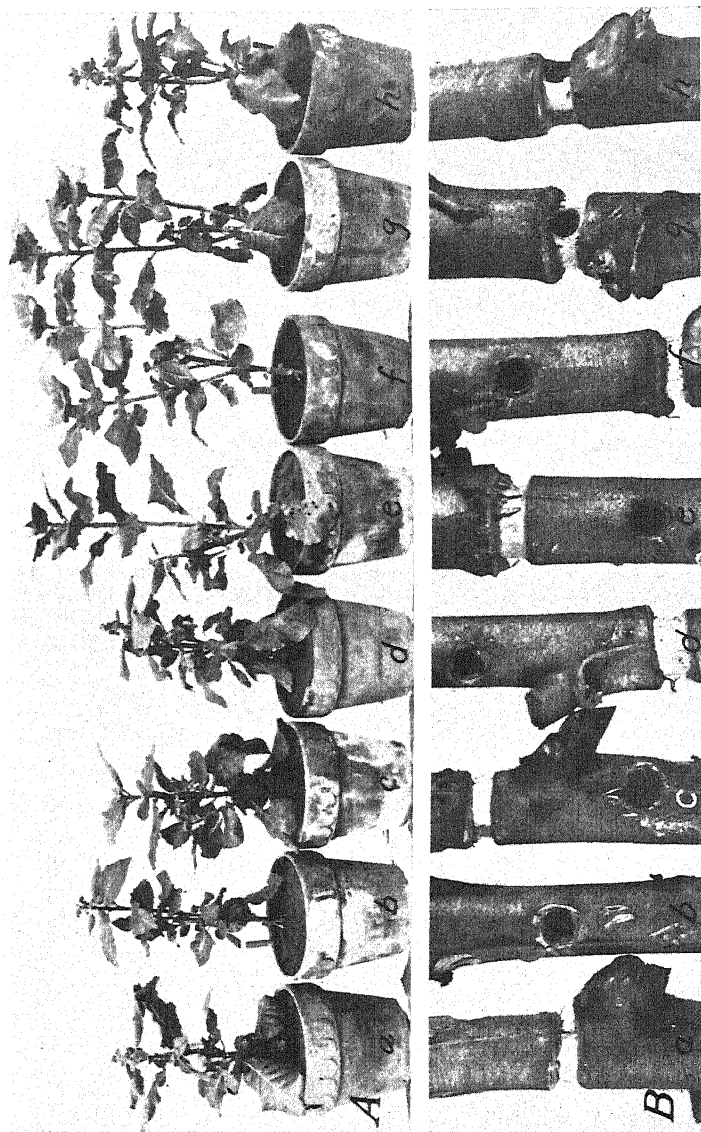
FIGURE 6.—*Nicotiana tabacum* plants after ringing. *A*, Series of plants showing results of interference with virus movement by certain types of rings. The virus moved past the rings in plants *a*, *b*, *c*, *d*, and *h*, and failed to move past the rings in plants *e*, *f*, and *g*, as shown by symptoms and lack of symptoms, respectively, on the shoots coming from below the rings. *B*, *a–h*, Ringed areas of plants shown in *A*. (Plants photographed 1 month after inoculation.)

several weeks, but curly-top symptoms appeared in no case in any part of the plant below the killed area.

Further experiments were made to determine the part of the stem through which the virus is able to move. Plants were grown to the beginning of flower-bud production. The stems were pruned back

to a height of 18 inches and buds were allowed to start.   The plants were then divided into 8 lots and treated as follows:

Lot 1.—A ring of bark was removed from the internode below the second or third bud from the top.   This is termed an external ring (fig. 6, *B*, *a*).

Lot 2.—A hole was made through the bark and wood of the internode about one half of an inch below the second or third bud, by means of a small cork borer and the pith and internal phloem were removed, exposing a ring of wood one fourth of an inch wide on the inner side of the woody cylinder.   This is termed an internal ring (fig. 6, *B*, *b*).

Lot 3.—An outer and inner ring 1 inch apart were made with the outer ring above the inner and the two rings separated by the second or third bud from the top of the plant (fig. 6, *B*, *c*).

Lot 4.—The plants were treated as in lot 3, except that the relative positions of the two rings were reversed (fig. 6, *B*, *d*).

Lot 5.—An outer and an inner ring, 1 inch apart, were made with the outer ring above the inner and both rings in the second or third internode (fig. 6, *B*, *e*).

Lot 6.—The plants were treated as in lot 5 except that the relative positions of the rings were reversed (fig. 6, *B*, *f*).

Lot 7.—Outer and inner rings were made in the second or third internode, the two rings being placed at the same level on the stem (fig. 6, *B*, *g*).

Lot 8.—Plants were treated as in lot 7, except that a small strand of bark was left in the outer ring (fig. 6, *B*, *h*).

These treatments were designed to determine whether the curly-top virus moves (1) through internal phloem, (2) through the external phloem, (3) from the internal phloem to the external phloem through unions of the two in the leaf traces, (4) from the external to the internal phloem through unions of the two in the leaf traces, (5) from the internal phloem to the external phloem through the medullary rays or other parts of the woody cylinder, (6) from the external phloem to the internal phloem through the medullary rays or other parts of the woody cylinder, (7) downward through the woody cylinder, or (8) through a very small strand of bark bridging an external ring.

Immediately after ringing, the top bud on each plant was exposed to viruliferous leaf hoppers.   Symptoms of curly top appeared on the inoculated bud in from 6 to 13 days.   Typical results of a series of tests are shown in figure 6, *A*, and results of all tests are given in table 5.

TABLE 5.—*Influence of rings on virus movement in plants of Turkish tobacco (Nicotiana tabacum) cut back to a height of 18 inches, inoculated in top two buds, and ringed 1 to 3 inches below the point of inoculation*

Number and position of rings	Plants infected	Effect on plants in which virus passed rings		Effect on plants in which virus did not pass rings	
		Plants affected	Average period between inoculation and appearance of symptoms	Plants affected	Average period between inoculation and death of parts above rings
	Number	*Number*	*Days*	*Number*	*Days*
Outer ring only	15	15	9.9	0	
Inner ring only	8	8	11.0	0	
Outer ring 1 inch above inner, bud between	15	15	9.6	0	
Outer ring 1 inch below inner, bud between	15	15	11.1	0	
Outer ring 1 inch above inner, both in internode	11	4	35.0	7	83.8
Outer ring 1 inch below inner, both in internode	16	3	27.0	13	97.5
Outer and inner rings at same level	14	0		14	86.9
Outer and inner rings at same level, strip of bark in outer ring	7	7	10.4	0	

In all cases where there was an uninterrupted channel through either internal or external phloem across the rings the virus moved downward with little apparent delay. In many instances in some types of ringing, symptoms were evident on shoots below the rings before any sign of disease appeared on the inoculated parts. In the type of ringing in which the appearance of virus below the ring was dependent on its movement through a distance of less than one fourth of an inch of woody cylinder, no symptoms appeared in any parts below the rings in any of the 14 plants inoculated. The diseased parts above the rings lived for an average period of 86.9 days from the time of inocu-
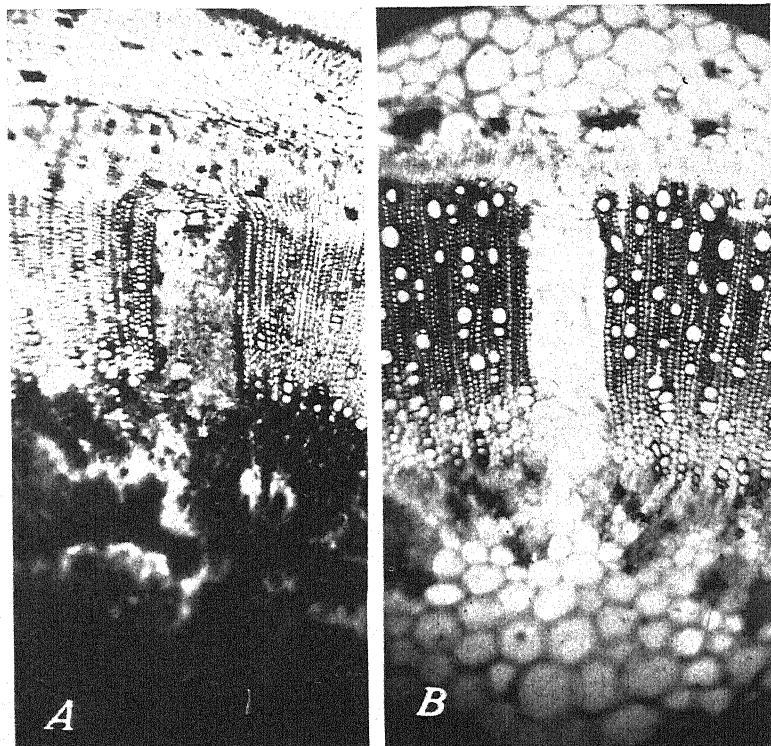
FIGURE 7.—Regeneration of cells in the woody cylinder of tobacco plants having two rings 1 inch apart in an internode: *A, Nicotiana tabacum; B, N. glauca.* × 40.

lation. In the series of tests in which the two rings were at different levels in the internode and the external ring was the higher, the virus was held in the inoculated parts in 7 of the 11 plants used. The inoculated parts in these 7 plants lived an average period of 83.8 days. In four plants symptoms appeared below the rings in an average period of 35 days. In the series in which both rings were at different levels in an internode and the internal ring was the higher, the virus was held in the inoculated parts in 13 of the 16 plants used, the average life of tops of the 13 plants being 97.5 days. In three plants symptoms appeared in parts below the rings in an average period of 27 days. In series in which there was an uninterrupted path of phloem across the rings, symptoms appeared on the parts below rings in an average period of 9.6 to 11.1 days, which differed in different series.

The seven plants in which symptoms appeared below rings in the two series in which the rings were at different levels in an internode were examined to determine what conditions were present or what changes had occurred that might have a bearing on an explanation of virus passage.   Serial sections of entire internodes revealed definite regions of regeneration of what appeared to be medullary rays in one or more areas of the woody cylinder.   These regions were sufficiently evident to be recognizable to the unaided eye and seemed to consist of masses of newly formed tissue extending from a region of very active internal phloem outward and downward to the bark, splitting the wood in their growth.   These interphloem strands seemed to have their origin just above the top of the internal ring.   It is considered probable that the internal phloem transports considerable food under normal conditions but that since the internal phloem is enclosed by a rigid tissue the opportunity for the expression of growth impulses may be limited, and the tendency to initiate regeneration of medullary rays may be greater than from the opposite side of the woody cylinder, where the food brought down in the external phloem is used to form a great amount of callus, wood, bark, and roots.   A typical regeneration area is shown in figure 7, *A*.   These areas of regenerated tissue were examined microscopically for phloem elements.   They were found to be composed of a large variety of active cells of different shapes and sizes, some narrow and very much elongated, others broader but rectangular, and many oval or irregular.   Phloem tissue could not be identified definitely, but it seems quite probable under the circumstances that tissue capable of translocating elaborated foods was present in these connecting strands.

NICOTIANA GLAUCA

To have available a more woody stem, a perennial species of tobacco, *Nicotiana glauca*, was chosen for several series of ringing experiments similar to those described for *N. tabacum*.   *N. glauca*, however, has not been reported as susceptible to curly top, and before the ringing experiments were started preliminary tests were made to determine the reaction of the species to the curly-top virus.   To test susceptibility to infection, large numbers of viruliferous leaf hoppers were allowed to feed on five small plants for several days.   None of these plants developed symptoms of curly top.   Larger plants, 3 feet or more in height, were then used.   These were pruned to a height of about 18 inches, and a 3-inch length of stem from a plant of *N. tabacum* affected with curly top was grafted at the top.   The *N. tabacum* scions grew well and developed typical curly-top symptoms.   No signs of disease appeared, however, on any of the new growth from the stock.   Parts of stems from healthy *N. tabacum* plants were then grafted in at the base of the *N. glauca* stems at points about 14 inches below the diseased scion.   These grafts without exception became diseased, showing that the *N. glauca* plants were infected and that the virus had moved downward through approximately 14 inches of stem.

It was later proved that *Nicotiana glauca* is susceptible to infection by direct feeding of leaf hoppers.   Healthy *N. tabacum* stems were grafted into the base of *N. glauca* plants and viruliferous leaf hoppers were allowed to feed on young shoots of the *N. glauca* stems.   The virus passed from the inoculated shoots through the stem and infected the graft several inches below.

Tests were next made to determine how long the virus would remain active in *Nicotiana glauca*. Two plants which had been inoculated from *N. tabacum* by the grafting method were selected and all *N. tabacum* tissue removed. These two plants were tested at intervals over a long period by grafting portions of their stems on healthy *N. tabacum* plants. The production of disease in this latter species showed that the virus was active in *N. glauca* 2 years after the plants were infected. At no time, however, were signs of curly top visible on these plants. The species seems to be a symptomless carrier of the virus under the conditions of these tests.

The stem of *Nicotiana glauca* is very well adapted to ringing experiments, but since the plant does not show symptoms of the presence of virus it was necessary to modify the technic used in previous experiments with *N. tabacum*. A suitable modification was accomplished by grafting *N. tabacum* on *N. glauca* stems as follows: *N. glauca* plants were grown in 12-inch pots to a height of 3 to 7 feet and pruned to a height of 18 inches. Plants were inoculated by grafting infected *N. tabacum* stems at the top. A second portion of stems from *N. tabacum*, this part from a healthy plant, was grafted in at the base about 14 inches below the top or diseased graft. This latter graft was used as an indicator of the presence of virus in the basal portions of the *N. glauca* plants, and the *N. glauca* stem was used as a medium in which to study the influence of rings in the downward movement of virus.

The rings were placed on the *Nicotiana glauca* stems about 1 inch below the point of union with the upper graft of *N. tabacum*. The rings were made as described for *N. tabacum* with the additions shown in table 6, where the results of this experiment are tabulated.

TABLE 6.—*Influence of rings on virus movement in Nicotiana glauca plants cut back to a height of 18 inches and grafted to curly-top Turkish tobacco at top and to healthy Turkish tobacco at base*

Number and position of rings	Plants inoculated	Effect on plants in which virus passed rings		Effect on plants in which virus did not pass rings	
		Plants affected	Average period between inoculation and appearance of symptoms below rings	Plants affected	Average period between inoculation and death of part above rings
	Number	*Number*	*Days*	*Number*	*Days*
Neither inner nor outer rings	10	10	23.6		
Outer ring only	15	15	23.8		
Inner ring only	15	15	22.7		
Outer ring 1 inch above inner, bud between	15	15	25.5		
Outer ring 1 inch below inner, bud between	15	15	22.5		
Outer ring 1 inch above inner, both in an internode	15	3	63.0	12	245
Outer ring 1 inch below inner, both in an internode	15	1	33.0	14	261
Outer and inner rings at same level in an internode	15	0		15	203
Outer and inner rings at same level, strip of bark in outer ring	10	10	19.5		
Outer and inner rings at same level, strip of phloem in inner ring	5	5	26.2		
Outer ring 1 inch above inner, both in internode, strip of bark in outer ring	6	6	19.6		
Outer ring 1 inch below inner, both in internode, strip of bark in outer ring	5	5	20.0		

Plants having no rings required about the same average length of time for the appearance of symptoms on the lower graft as those in the groups having only an external or an internal ring.   Two rings 1 inch apart seemed to have no influence in delaying the passage of virus in plants where the two rings were separated by a bud, regardless of their relative positions.   The average length of time required for the appearance of symptoms was 25.5 days in plants where the outer ring was above the bud and 22.5 days in plants where the inner ring was above the bud.   Whether this difference of 3 days is significant may be questioned.   However, it is worthy of note that in the plants requiring the longer period for the appearance of symptoms on the lower graft the rings were so placed that materials moving downward in the phloem would be required to pass outward to the external phloem from the internal phloem through connection in the leaf traces.

Two rings, one external and the other internal, 1 inch apart, and both in an internode, prevented the passage of virus in 26 of 30 plants in the two series shown in table 6.   In these two series, the 4 plants in which the virus passed the rings were of the same age and had been inoculated and ringed at the same time.   At the time of inoculation they had relatively immature stems with thin woody cylinders. Serial sections of the internodes in which the two rings were located in all 4 plants revealed strands of newly formed tissue extending from the internal phloem at the top of the internal ring downward to the external phloem of the bark.   Figure 7, *B*, shows a section of one of these strands.

In 15 plants having two rings at the same level in an internode, there was no instance in which the virus passed the rings.   The average length of life of the diseased parts above the rings was 203 days. Figure 8, *A*, shows a plant of this series that retained virus above the ring for more than a year with no movement across the ring during this time.

The presence of a small strand of either internal or external phloem bridging rings which otherwise prevented virus passage, permitted the virus to pass with no measurable delay.   The influence of a small strand of bark as compared with complete severing of phloem continuity is illustrated in figure 9, *A* and *B*.   The influence of a bud between the internal and external rings is illustrated in figure 10, *A* and *B*.

<center>RATE OF VIRUS MOVEMENT</center>

A better understanding of the movement of virus in plants and of the factors influencing it would throw new light on some of the fundamental problems presented by plant viruses.   The subject of virus movement in plants has received attention from several investigators. Sufficient evidence has been accumulated to indicate considerable variation in the behavior of different viruses.   Whether this variation is due to the specific nature of the viruses or to the functioning of the plants in which they occur is a question of considerable interest and importance.

McCubbin and Smith (*18*) were among the first to present data on the rate of dispersion of viruses in plants.   They measured the rate of movement of the virus of tomato mosaic by layering tomato plants, inoculating them at the distal end of one of the branches, and severing the stems between the rooted portions at different distances from the

point of inoculation at known time intervals. By this means they were able to calculate a rate of movement of 1 to 2 inches per day or 1 to 2 mm per hour.

Severin (22) inoculated the distal ends of beet leaves with the curly-top virus by means of leaf hoppers and severed the inoculated
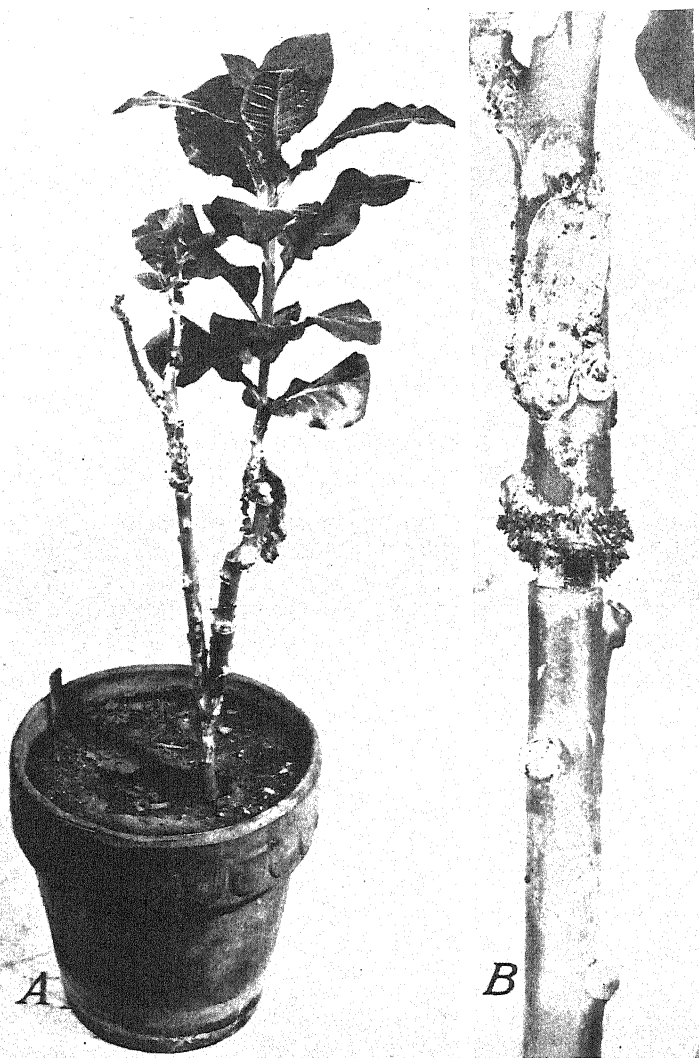
FIGURE 8.—*A*, *Nicotiana glauca* plant that had a diseased *N. tabacum* graft placed at the top and a healthy *N. tabacum* graft placed at the base and rings of internal and external phloem removed at the same level immediately below the top graft. After 420 days, virus was present in the part above the rings and absent in the parts below the rings. *B*, Ringed area, natural size. (Photographed 1 year after grafting.)

leaves at measured distances from the point of inoculation after different time intervals. The most rapid movement measured by this means was 7 inches in 30 minutes, or a rate of 14 inches per hour.

Storey (27), using methods similar to those of Severin, found that in 3 of 8 plants the virus of maize streak moved downward from the

point of inoculation at the distal end of a maize leaf, a distance of 40 cm, in 2 hours.

Böning (4) found that the virus of tobacco mosaic moved 13 cm in 2 days in tobacco and 9 cm in 2 days in tomato.

Holmes (15), although not attempting an accurate measurement of maximum virus movement, has presented some very interesting studies on rate of invasion of tobacco plants by the mosaic virus.
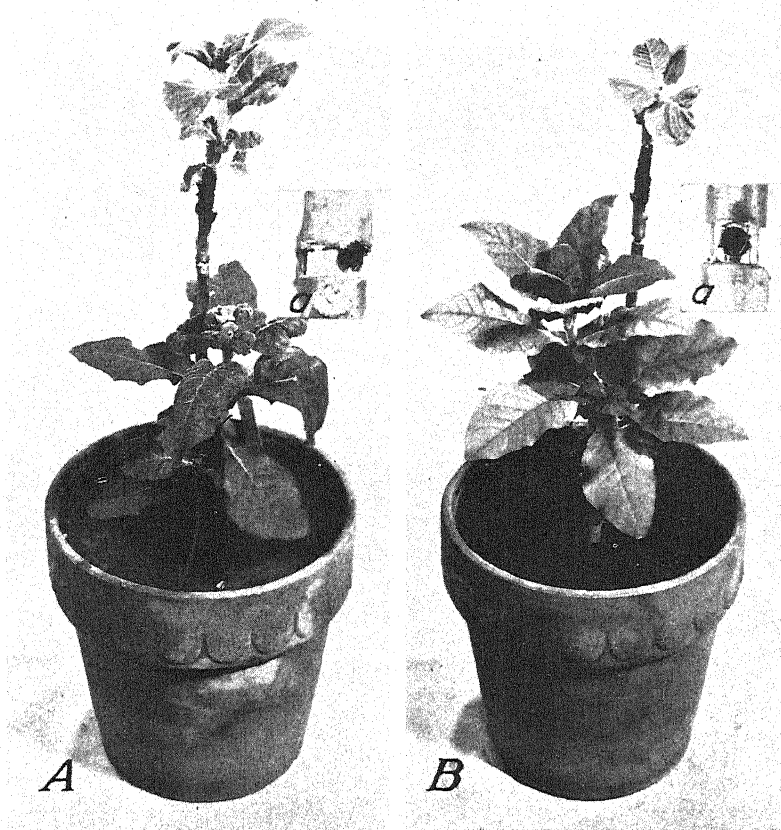
FIGURE 9.—Effect of a small strand of bark on the downward movement of virus past rings in *Nicotiana glauca*: A, Plant having internal and external rings at same level and a strip of bark in the outer ring Curly-top symptoms may be seen on the lower graft. B, Plant having rings as in A but no strip of bark in the outer ring. The lower graft shows no curly-top symptoms. A, a, and B, a, Rings of respective plants, natural size. (Photographed 40 days after inoculation.)

He shows that the rate is at first very slow until the vascular bundle is reached. The virus moves more rapidly along the veins traversing the leaf blade and petiole. Apparently, the speed of movement is again accelerated when the virus passes into the stem from the inoculated leaf.

These results indicate a wide range in rate of movement among viruses in affected plants. This range extends from a rate of 1 to 2 inches per day in the tomato mosaic virus to a rate of 14 inches per hour in the virus of curly top. The factors determining these wide differences are of interest. Among the factors that may exert an

influence at the time of testing are the specific nature of the tissue inoculated, the environmental conditions, the physiologic tone of the plant, and the species or variety of the plant. An effort has been made to extend the work done by Severin and to make further
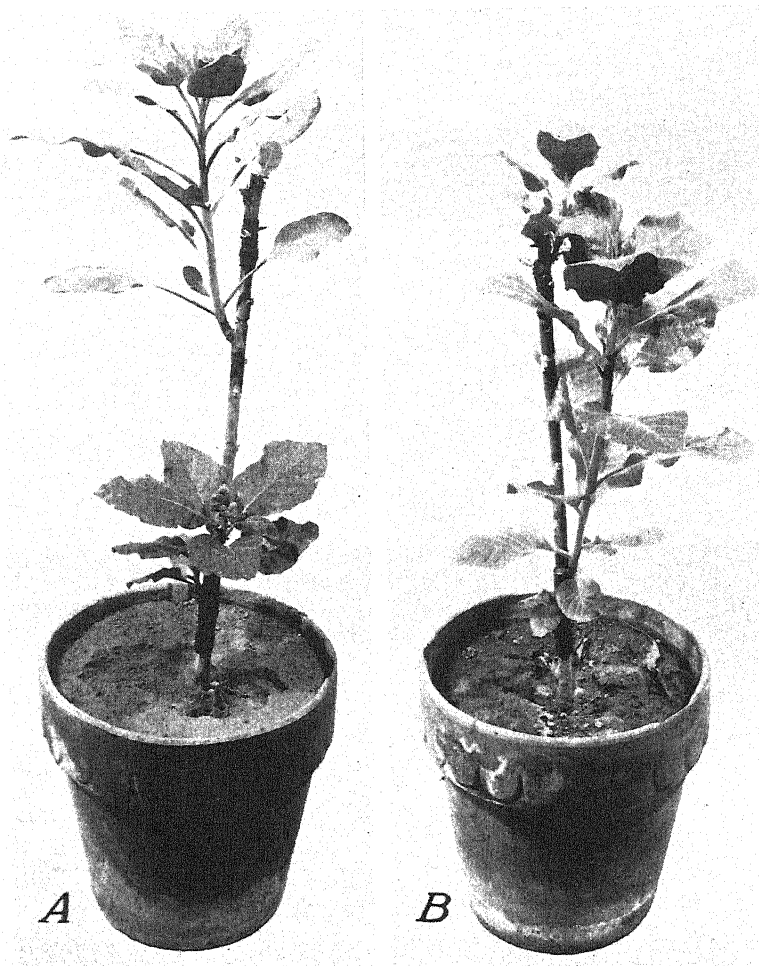
FIGURE 10.—Effect of a bud between internal and external rings on the passage of virus downward in a stem of *Nicotiana glauca: A*, Plant having external and internal rings and bud between. Note symptoms on the lower graft. *B*, Plant having internal and external rings in an internode. Note the absence of symptoms on the lower graft. (Photographed 40 days after inoculation.)

determinations on the rate of movement of the curly-top virus. Tobacco and sugar beet were used in these experiments.

TOBACCO

Measurements were made of the rate of the downward movement of virus in the stems of the Turkish variety of *Nicotiana tabacum*. All the plants were more than 24 inches high at the time of their selection for this experiment, and some of them were showing the first

indication of blossom buds. The youngest leaves of each plant were enclosed in a celluloid cage into which 50 to 100 viruliferous leaf hoppers were placed. The leaf hoppers were allowed to feed 5 hours.

The inoculated plants were incubated for different periods of time. At the end of the period allowed for virus movement they were cut off at a point 24 inches below the lowest point of inoculation, and all leaves were removed except the small ones on which the leaf hoppers fed. The stem was next cut into eight parts, each 3 inches long, and the segments placed in sand. In nearly all cases these cuttings rooted readily and produced a very satisfactory growth. The appearance of curly-top symptoms on a cutting indicated that the virus had reached that particular part of the stem in the period allowed for downward movement. The results of this experiment are shown in table 7. The virus did not move out of the inoculated 3 inches of the stem in any plant in 24 hours. In 48 hours the virus moved a distance of 24 inches in plant 10, or at a rate of one half inch per hour. The extent of stem invasion increased irregularly with the period allowed for virus movement up to 144 hours, when the virus had in all cases moved through the full 24 inches of stem and had reached the root of the plant. As calculated from these data, the maximum rate of virus movement is one half inch per hour (plant 10) for a 48-hour period.

Table 7 shows that there were decided differences among individual plants. For example, in plants 16 and 19 the virus had not moved out of the inoculated 3 inches in 96 hours, whereas in plant 17 it moved 24 inches in the same length of time. Perhaps the most interesting results were obtained from plant 20, in the 96-hour incubation period and from plants 23 and 24, in the 120-hour period. In plant 20 sections 1, 2, 3, 5, and 6 and the root portion were diseased and sections 4, 7, and 8 were healthy. In plant 23 sections 7 and 8 were diseased, whereas all the other sections were healthy, including the inoculated tip and the root below the 24-inch mark. In plant 24 sections 4, 5, and 7 were healthy and all the other sections were diseased. In all these plants, as usual in the experiment, the sections recorded as diseased showed symptoms on the first leaves from the buds and continued to show marked symptoms so long as they lived or until they were discarded. All the cuttings from these three plants were transferred to soil in 8-inch pots and the healthy-appearing ones were grown to flowering. The plants were then further tested for the presence of virus by grafting portions of the stems on healthy plants. In no case was evidence of the presence of virus obtained in plants grown from sections which had shown no symptoms. Symptoms were present at all times in all plants grown from other sections.

The significance of this erratic distribution of virus is not clear. In plants 20 and 24 it might well be that in its movement downward the virus failed to come into contact with tissue in segments 4, 7, and 8, and in segments 4, 5, and 7, respectively, in which it could become established and multiply. In plant 23, however, it is diffcult to account for the absence of virus in the inoculated portion and its presence in two segments farther down the stem.

TABLE 7.—*Rate of downward movement of curly-top virus in stems of Turkish tobacco* (Nicotiana tabacum)

Plant no.	Period between inoculating and making cuttings	Infection [a] of indicated 3-inch cutting [b] and root								
		First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Root
	Hours									
1	24	+	—	—	—	—	—	—	—	—
2	24	+	—	—	—	—	—	—	—	—
3	24	+	—	—	—	—	—	—	—	—
4	24	+	—	—	—	—	—	—	—	—
5	24	+	—	—	—	—	—	—	—	—
6	48	+	—	—	—	—	—	—	—	—
7	48	+	+	+	—	—	—	—	—	—
8	48	+	+	+	—	—	—	—	—	—
9	48	+	—	—	—	—	—	—	—	—
10	48	+	+	+	+	+	+	+	+	+
11	72	+	+	—	—	—	—	—	—	—
12	72	+	—	—	—	—	—	—	—	—
13	72	+	—	—	—	—	—	—	—	+
14	72	—	—	—	—	—	—	—	—	+
15	72	+	+	+	+	+	+	+	+	+
16	96	+	—	—	—	—	—	—	+	—
17	96	+	+	+	+	+	+	+	+	+
18	96	+	+	+	+	+	+	+	+	+
19	96	+	—	—	—	—	—	—	—	—
20	96	+	+	+	—	+	+	—	—	+
21	120	+	+	—	+	+	+	—	—	—
22	120	+	+	+	+	+	+	+	—	—
23	120	—	+	—	—	—	+	+	+	—
24	120	+	+	—	—	+	—	+	+	+
25	120	+	+	+	+	+	+	+	+	+
26	144	+	+	+	+	+	+	+	+	+
27	144	+	+	+	+	+	+	+	+	+
28	144	+	+	+	+	+	+	+	+	+
29	144	+	+	+	+	+	+	+	+	+
30	144	+	+	+	+	+	+	+	+	+

[a] Plus and minus signs indicate positive and negative results, respectively, obtained from planting various 3-inch sections of the inoculated stem. Where a cutting showed infection it was considered to indicate that the virus had reached that section of the stem in its downward movement.
[b] Counting downward from inoculated top.

SUGAR BEET

Seedling Beets

Experiments with seedling beets were planned with a view to studying the rate of movement of virus in cotyledons under different conditions of temperature. Seedling plants were used that had the beginning of first true leaves and cotyledons more than 1 inch long. Leaf hoppers were caged singly on the tip of one cotyledon of each plant. The time at which each leaf hopper started to feed was noted and the feeding period was terminated after the desired interval by removing the leaf hopper. The plants were grown in 6-inch pots, 4 plants per pot. The pots were numbered consecutively. In all even-numbered pots the cotyledons on which the leaf hoppers fed were severed 1 inch from the point of feeding after the desired period allowed for virus movement. The odd-numbered pots were kept as controls and received the same treatment as the even-numbered pots, except that the cotyledons on which the leaf hoppers fed were not removed. The feeding periods of the leaf hoppers were 2, 3, and 5 minutes; the periods allowed for virus movement were 2, 3, 5, 10, and 15 minutes; and the air temperatures at which the various tests were made were roughly 60°, 85°, 110°, and 135° F. The results of these experiments are shown in table 8.

TABLE 8.—*Rate of movement of virus in cotyledons of young sugar-beet plants*

Temperature (°F.)	Leaf-hopper feeding period	Period allowed for virus to move 1 inch	Cotyledon removed			Cotyledon not removed		
			Plants inoculated	Plants infected		Plants inoculated	Plants infected	
	Minutes	*Minutes*	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*
60	2	2	80	0	.9	79	0	.0
	3	3	80	0	.0	80	0	.0
	5	5	80	1	1.2	80	9	11.2
	5	10	66	5	7.5	71	11	15.4
	5	15	77	5	6.4	82	6	7.3
85	2	2	176	0	.0	183	9	4.9
	3	3	81	15	18.5	79	24	30.3
	5	5	70	8	11.4	76	19	25.0
	5	10	74	14	18.9	74	17	22.9
	5	15	73	13	17.8	75	21	28.0
	5	60	80	11	13.7	80	15	18.7
110	2	2	80	2	2.5	80	8	10.0
	3	3	78	6	7.7	80	11	13.7
	5	5	82	32	39.0	78	31	39.7
	5	10	68	18	26.4	64	12	18.7
	5	15	80	26	32.5	80	25	31.2
135	2	2	80	1	1.2	80	6	7.5
	5	5	40	8	20.0	40	10	25.0

Infection did not occur in any plant after the 2- and 3-minute feeding periods in the 60° series nor in any plant after the 2-minute feeding in the 85° series. Infection occurred in test plants and controls after all the other feeding periods and exposures in all series. As calculated from these data, the rates of movement were as follows: At 60°, 12 inches per hour; at 85°, 20 inches per hour; at 110°, 30 inches per hour; and at 135°, 30 inches per hour. However, considerable caution should be exercised in drawing conclusions from these calculations regarding the effect of temperature on the rate of virus movement. Although the calculated rate was lowest at 60°, the percentage of infection was also low. The low percentage of infection indicates a lower inoculative efficiency on the part of the vector, which in turn may mean that the minimum time required for infection to occur may be longer than at higher temperatures, thus correspondingly reducing the available period for virus movement. However, the results at 110° seem to furnish some support for the assumption that the virus moves more rapidly at this temperature than at 60° or at 85°, since in the 5-, 10-, and 15-minute exposures, as measured by the controls, the removal of the cotyledons on which the leaf hoppers fed seemed to have little influence on the percentage of plants which later developed disease; whereas at 60° and 85° the percentage of infection was considerably reduced by removing the cotyledons after exposure.

BEET PLANTS HAVING SEVERAL TRUE LEAVES

In seedling plants the determination of very rapid rates of virus movement is limited by the minimum duration of the required infection period and by the length of the cotyledons. In order to obtain a relation between time of infection and extent of movement which would permit the detection of rates of movement more rapid than 30 inches per hour, plants having leaves 3 to 10 inches long were used in a second experiment. Ten leaf hoppers were allowed to feed 6 minutes at the distal end of a leaf on each plant at a temperature of 85° to 100° F. In the odd-numbered pots the inoculated leaves were severed 1, 2, 3, etc., up to 10 inches from the point of inoculation 6

minutes after feeding started. The even-numbered pots were retained as controls. The experiment was run in three series (table 9). The results within a series are considered comparable, but one series is not strictly comparable with any other, because of differences in date of inoculation and in age of plants used.

The results of this experiment show a maximum downward movement of 6 inches in 6 minutes, or a rate of 60 inches per hour. An analysis of results indicates that even this rapid rate of movement does not represent the maximum rate attainable under the most favorable conditions for movement. Series 1, 2, and 3 of table 9 show considerably less infection in plants from which the inoculated leaf was removed than in the controls. This, however, did not hold in some of the subsequent tests made on very rapidly growing plants.

Experiments 1 and 2 of table 9 show the results of two additional tests on plants growing at different rates at the time of inoculation. The plants used in experiment 1 of this table were thrifty but were not growing at an excessive rate; those used in experiment 2 were in rich soil and were growing very rapidly. In experiment 1, infection in the test plants was considerably less than in the controls. Moreover, infection decreased as the distance of required virus movement increased. The results of this experiment, if standing alone, would indicate (1) that there was a rather uniform decrease in infection as the length of leaf removed was increased and (2) that the virus would not move more than 4 inches in 6 minutes.

TABLE 9.—*Virus movement in leaves of young sugar-beet plants*

Series or experiment	Length of leaf removed	Inoculated leaf not removed			Inoculated leaf removed			Calculated rate of virus movement per hour
		Plants inoculated	Plants infected		Plants inoculated	Plants infected		
	Inches	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*	*Inches*
Series 1	1	120	56	46.6	120	27	22.5	10
	2	120	64	53.3	120	33	27.5	20
	3	120	50	41.6	120	20	16.6	30
Series 2	4	140	38	27.1	140	15	10.7	40
	5	140	46	32.8	140	15	10.7	50
	6	140	47	33.5	140	17	12.1	60
Series 3	7	40	10	25.0	40	0	0	
	8	40	11	27.5	40	0	0	
	9	40	10	25.0	40	0	0	
	10	40	10	25.0	40	0	0	
Experiment 1	1	20	17	85	20	8	40	10
	2	20	13	65	20	6	30	20
	3	20	15	75	20	3	15	30
	4	20	11	55	20	1	5	40
	5	20	7	35	20	0	0	
	6	20	6	30	20	0	0	
Experiment 2	1	20	13	65	20	8	40	10
	2	20	14	70	20	10	50	20
	3	20	10	50	20	10	50	30
	4	20	13	65	20	11	55	40
	5	20	12	60	20	11	55	50
	6	20	12	60	20	9	45	60

In rapidly growing plants, results were quite different. The removal of the inoculated leaf after 6 minutes did not appreciably decrease the percentage of infection as measured by the controls. This was true regardless of whether 1, 2, 3, 4, 5, or 6 inches of leaf was removed, and the chances, therefore, that the virus would move 6 inches in 6 minutes were about the same as that it would move 1 inch in 6 minutes. It seems probable that under the conditions of this experiment virus could have been shown to move more than 6 inches in 6 minutes had longer leaves been available.

DISCUSSION

The results obtained in the various experiments outlined in this paper are interpreted as indicating a very intimate relationship between the curly-top virus and the phloem tissue of affected plants. Virus is introduced into the phloem by its insect vector. It moves downward from the point of inoculation toward the root system at a very rapid rate. Results of leaf-hopper feeding experiments indicate that the virus concentration in exudate, believed to be derived largely from the phloem, is high as compared with that in expressed juice from the entire beet. Virus apparently does not pass from the xylem into the phloem or any other tissue in sufficient quantities to cause systemic infection. After it is established in the phloem the evidence indicates that it passes into adjacent parenchyma tissue only in very limited amounts in the beet, and experiments have failed to demonstrate that it ever occurs in the parenchyma of two species of tobacco. The evidence supporting these conclusions may be worthy of further study.

It is considered probable that the close association of the virus with the phloem tissue may have a bearing on the difficulty encountered in obtaining infection by mechanical inoculation. The ordinary methods of mechanical inoculation undoubtedly can be depended upon to introduce virus into parenchymatous tissue of various kinds, since infection is induced readily with other viruses by these methods. Artificial-inoculation experiments indicate that introduction of virus into such parenchyma cells as the leaf hairs of beet, tobacco, squash, or bean, never produces infection. Since the virus does not set up a systemic infection after its introduction into parenchymatous cells, only two other possible courses are open to it. It must either remain in these cells and fail to set up a systemic infection or it must be inactivated by substances resulting from cell injury or by normal constituents of parenchymatous cells.

In the light of the information now available, it seems probable that if artificial infection is to be obtained in any appreciable percentage of the plants inoculated the virus must be introduced directly into the phloem. Clearly the introduction of virus into phloem by mechanical means is attended with difficulty. Needles that are available for introducing virus into plants are large enough to crush many cells, and it is probable that the phloem is so badly injured in the process of inoculation that the virus does not often become established. Even if needles sufficiently fine to penetrate the phloem without causing excessive injury were available, they would probably still fall far short of the insect vector in effectiveness. Such a needle would necessarily carry the virus on the surface, where it would be exposed to the action of contents of parenchyma cells and to depletion in the passage of the needle through tissue exterior to the bundle. Once the needle penetrated the phloem, a portion of the virus might be liberated. If the needle were withdrawn, the phloem content, being under a positive pressure, would pass quickly into the cavity that was left, probably carrying a part or all of the virus deposited in the phloem back into the region of parenchyma.

The best available method of artificial inoculation seems crude when compared with the refinements introduced by the insect vector. The leaf hopper's stylets are extremely slender and seem to find the

phloem with remarkable accuracy. As the stylets pass through the parenchymatous regions exterior to the phloem, a protective sheath is laid down which may effectually exclude contents of surrounding cells. After the phloem is punctured the insect remains in a feeding position for appreciable periods and may leave considerable deposits of sheath material in the vascular area. As the stylets are removed the puncture is probably completely plugged by sheath material.

Since virus is introduced into the phloem directly by the leaf hopper, its movement to certain parts of the beet plant probably starts immediately and is very rapid. Probably the phloem of the growing point is invaded in a few hours and the entire phloem network of the plant is invaded in a few days. Evidence indicates that under some conditions the virus is closely restricted to the phloem, under other conditions it may escape into the intercellular spaces of the parenchyma. This is shown by the occurrence of drops of exudate having a high virus content on the petioles of badly diseased beets. Esau (*13*) has shown that this exudate moves from the vicinity of the phloem to the exterior of the petiole through the intercellular spaces. The causes of such movements are not clearly understood.

Crafts (*9*) states that the phloem is normally under a positive pressure and suggests that the cambium on one side and the starch sheath on the other limit lateral movement of phloem content. It is possible that phloem necrosis and the presence of regenerative tissue may interfere with the movement of solutes and increase the pressure in the phloem of diseased beets. This increased pressure may be sufficient to force phloem content through the limiting layers into the intercellular spaces of the parenchyma and to the surface of the petiole, or, as Esau has suggested, the presence of the virus may render the limiting layers more permeable to phloem content. If the cells of the limiting layer are rendered more permeable it seems probable that there might be a seepage of virus from phloem tissue throughout the plant. If the virus is not extruded through breaks in the limiting layer it may pass through cells that are rendered more permeable. Whether the virus is able to pass from the intercellular spaces into the cells of the parenchyma seems doubtful. The failure to obtain infection by introducing virus into parenchyma cells and the rapid inactivation of virus in expressed juice point to parenchyma as being a very poor medium for virus. The failure of leaf hoppers to acquire virus from any type of parenchyma except that immediately below the crown and in one instance from parenchyma of the petiole indicates that the virus content of parenchyma is very low. The relatively small amount of virus obtained by leaf hoppers from expressed juice as compared to that obtained from phloem exudate indicates that the juice is low in virus content or that the leaf hoppers are much less efficient in acquiring virus from expressed juice than from phloem exudate.

The weight of evidence seems to indicate that the virus content of parenchyma of beet is very low. The virus present in parenchyma may occur largely or exclusively in the intercellular spaces.

It seems probable that the virus may be even more closely restricted to the phloem in the two species of tobacco tested than in sugar beet. This is indicated by results of experiments in which the virus was held in inoculated parts in plants in which its further dispersal was dependent upon its ability to pass through a small amount of parenchym-

atous tissue. In most instances this parenchymatous bridge was composed of only a few cells, as in plants in which two rings, an internal and an external, 1 inch apart, were placed in an internode. In plants having the internal ring above the external the virus would move down the stem in the external phloem 1 inch past the internal ring, thus affording an area equal to the linear distance between the rings multiplied by the circumference of the internal ring for inward movement of virus through the wood or medullary rays to the internal phloem in which it could pass on down the stem. In plants having the positions of the two rings reversed the required movement would be from the internal to the external phloem through the wood or medullary rays. When it is considered that in these experiments active virus was held in the parts above the rings, in some instances for more than a year, with no leakage whatever of virus across the rings, the effectiveness of this barrier is apparent. To explain this phenomenon it is necessary again to assume that the virus does not pass out of the phloem or that it is very quickly inactivated by contact with normal cells of other tissues.

The close association of the curly-top virus with phloem tissue may have an important bearing on the failure of beet seeds to transmit disease. Artschwager (1) has shown that in the development of the beet seed there is no direct vascular connection between the mother plant and the young sporophyte. Therefore, materials that enter the embryo must do so by diffusing through a layer of meristematic or parenchymatous tissue. Results of experiments showing a lack of movement of virus through meristem and parenchyma indicate that such a layer would offer a formidable barrier to passage of the virus into the embryo, and might easily account for the absence of seed transmission of this disease.

Just how far it is safe to venture in applying this principle to other virus diseases is difficult to say. It would seem to go far toward explaining lack of seed transmission in all diseases in which the virus is restricted to the phloem. It may be important for other virus diseases. Nelson (20) has suggested such an explanation for the erratic transmission of bean mosaic. The virus of this disease is not known to be restricted to the phloem. It occurs in only a part of the seeds from diseased plants. In studying the development of the bean seed, Nelson found no direct vascular connection between the mother plant and the embryo. He suggests that the virus may be able to pass into the embryo through the nonvascular-containing layer only when this layer is in a meristematic stage of development. The failure of certain seeds to carry virus is accounted for by assuming an incomplete distribution of the virus in the phloem. Even with a complete virus invasion of the phloem, which may seem more likely in plants grown from diseased seeds, it would seem reasonable to suppose that the passage of virus through meristem or parenchyma of this type may be a hazardous one and successfully accomplished only under certain conditions. It is possible that the meristematic or parenchymatous layer of tissue separating the mother plant from the embryo may offer a structural or chemical barrier to virus passage even with such viruses as that of tobacco mosaic, which is known to occur in certain types of parenchymatous tissue.

The rate of movement of virus in sugar-beet leaves is so rapid as to call for a consideration of the mechanics of this phenomenon. Since it seems fairly obvious from data already presented that this movement

occurs in the phloem, the possibilities inherent in the solution of this problem are all the more interesting. Under conditions of the greatest measured rate of virus movement in sugar beet (6 inches in 6 minutes) it seems safe to conclude that there is no appreciable multiplication of virus in the plant in so short a time, and it would not seem that multiplication would be an appreciable accelerating force in movement. That such movement may result from any autonomous effort on the part of the virus particles seems out of the question, since this rate is several times greater than the most rapid movement of the swiftest moving micro-organisms known. Simple diffusion is infinitely slower than the measured rate of virus movement. Diffusion accelerated by protoplasmic streaming in the sieve tubes could account for no rate approaching maximum movement.

Certainly none of the foregoing theories will account for this rate of movement, regardless of whether virus is considered to be a living organized entity or a chemical compound. In spite of these facts it is now pretty definitely known to physiologists that certain substances move in phloem at a very rapid rate of speed. Mason and Maskell (19) calculate a rate of movement of sugars in the cotton plant equal to the rate of diffusion of molecules of that size in air. Crafts (10), using figures derived from growth increase in pumpkin and cucumber over a stated period, estimates an average linear rate of movement of 0.292 and 0.235 cm per minute, respectively, of materials through the stem into the fruits. By cutting stems of cucumber and measuring phloem exudate, he found that a calculated rate of movement of phloem content varying between 3.64 and 8.62 cm per minute could be induced. Crafts (9), in discussing food translocation, suggests that a mass movement of elaborated food materials takes place in the phloem. This mass movement he conceives as being dependent on the creation of a pressure gradient due normally to the increased osmotic pressure in the more active photosynthetic areas of the plant. This high osmotic pressure accelerates the intake of water, which in turn increases the hydrostatic pressure in the phloem and causes phloem content to move to parts where the pressure is lower. In such a system each compound would not move independently of other compounds as in simple diffusion, but would move at a rate approximating that of the mass as a whole, assuming no selective interference in the path of movement.

Accepting this hypothesis of food translocation, we have available a hypothesis of virus movement which seems to satisfy all the known conditions of such movement. In a virus movement of 6 inches in 6 minutes it seems unlikely, as stated previously, that virus increase would be an important factor under any conditions. Therefore virus concentration during the first few minutes after introduction is probably very low, and the virus itself could not function to increase appreciably the osmotic concentration of materials at the point of introduction. However, if the hydrostatic pressure of the phloem at the point of virus introduction were already high because of an abundance of photosynthates, and these photosynthates were being transported at a very rapid rate, the introduced virus particles would be carried along at a rate corresponding to the rate of food flow, provided of course that mechanical interference were the same for each. Under such conditions the virus would move at the same rate and in the same direction as elaborated foods in the phloem and would in fact be an indicator of the rate and direction of food translocation.

SUMMARY AND CONCLUSIONS

Sugar-beet plants were induced by various treatments to take up juice of beets affected with curly top through the water-conducting channels, but since none of the plants so treated became infected, it is evident that the curly-top virus does not pass from the tracheae into cells or tissues where it can become established and initiate pathologic symptoms.

Several other methods of inoculation were tried with inocula prepared in different ways, but an appreciable percentage of infection was obtained only when the phloem exudate of a curly-top beet was used as the inoculum.

The vector of the curly-top virus, *Eutettix tenellus*, feeds on the leaf veins, and its mouth parts usually penetrate the phloem region. As the mouth parts are inserted the insect lays down a sheath of apparently gelatinous material which completely incases the stylets. This sheath may seal off all cells penetrated that are external to the phloem, and thereby protect the virus as it is passed into or drawn out of the phloem by the leaf hopper.

The higher mortality rate in groups of leaf hoppers on parenchyma tissue, as compared with mortality in groups on tissue containing vascular elements, indicates that parenchymatous tissue does not serve as a favorable source of food. However, leaf hoppers having access to parenchyma lived longer than those given neither food nor water, indicating that they extracted a certain amount of material from parenchyma. Leaf hoppers given access to parenchyma tissue of petioles and crowns and to pith and immature seeds, all from diseased beets, and then caged in groups of 5 on healthy beet seedlings, infected only 10 of 428 plants. An equal number of insects from tissue containing vascular elements infected 210 of 428 plants. These results supplement other evidence supporting the view that virus is concentrated in the phloem and is present only in relatively small amounts in the parenchyma. Since in sugar beets affected with curly top, phloem content escapes into the intercellular spaces of parenchyma tissue surrounding the vascular bundles, it is suspected that the virus recovered from the parenchyma may have been derived from escaped phloem content.

The exudate occurring on the petioles and blades of beets affected by curly top and that from the cut surface of affected beets has a high virus content. Evidence indicates that the exudate that occurs naturally on the petioles and blades is derived largely from the phloem and that the exudate from the cut surface of the beet is derived from the phloem except as it may be contaminated by an undetermined amount of material from the xylem and from injured cells mainly parenchymatous in nature.

Healthy sugar-beet and tobacco plants were infected by grafting diseased plants on them. Beets were not infected when union of the grafted plants did not result. In the grafted tobacco plants a definite union consisting of meristematic tissue was found after 3 days. In unions 7, 8, and 9 days old, tracheal elements were apparently mature and strands of elongated cells paralleling the tracheae probably included functional phloem. No infection resulted until the seventh day. Infection increased from 27 percent on the seventh day to 100 percent on the twelfth day. In view of the fact that the virus does not gain effective entrance through the tracheae and did not in these

experiments pass through meristematic or parenchymatous tissue, it must have passed the graft union through the phloem elements.

Ringing experiments with *Nicotiana tabacum* and *N. glauca* showed that the virus passes all rings bridged by an uninterrupted path of phloem, internal or external or internal and external combined. The virus failed to move past the rings when the internal and external phloem were removed at the same level. Interruption of phloem continuity by rings placed at different levels in an internode prevented the passage of virus in 20 of 27 *N. tabacum* plants and in 26 of 30 *N. glauca* plants. Serial sections of the ringed area in all the plants in which the virus passed the rings showed in each case one or more areas of regenerated tissue connecting the internal and external phloem through the woody cylinder. In view of these findings it seems probable that virus does not move longitudinally or radially through any of the normal elements of the woody cylinder and that dispersal in these two species of tobacco is dependent on the presence of continuous phloem elements.

The movement of the curly-top virus in tobacco is relatively slow as compared to the movement in sugar beet. The fastest movement observed in tobacco was downward from the point of inoculation at the top of the plant to a point 24 inches below in 48 hours; a rate of movement of one half inch per hour.

In sugar beet the virus moves at a much more rapid rate. At air temperatures of approximately 85°, 110°, and 135° F. the virus moved outward in cotyledons from the point of inoculation a distance of 1 inch in 2 minutes. In larger beets the virus moved downward from the point of inoculation at the distal end of a leaf to a point 6 inches below in 6 minutes, a rate of movement of 60 inches per hour. These rapid movements of virus evidently occur in the phloem and it is suggested that they indicate a rapid translocation of plant materials. For these reasons virus may prove useful as an indicator in studies on the movement of elaborated foods.

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CHEMICAL COMPOSITION AND YIELD OF THE ALASKA PEA AS INFLUENCED BY CERTAIN FERTILIZERS AND BY THE STAGE OF DEVELOPMENT [1]

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INTRODUCTION

It is believed by many canners and growers of peas for the cannery that if potash in appreciable amounts is present in the fertilizer applied to a pea crop the peas will mature earlier and be harder, more starchy, and of lower quality at a given stage of development than if no potash or only a small amount is used. Boswell (*3, 4*) [2] conducted fertilizer experiments in three different parts of Maryland over a period of 3 years and used, among other materials, muriate of potash alone and in combination with nitrogen and phosphorus. All treatments were duplicated each year. Although he made no chemical analyses of the peas, field observations led him to conclude (*4, p. 352*) that—

Muriate of potash, alone or in combination with nitrogen and phosphorus, has given no consistent results with respect to yield, rate of maturity, or apparent condition of the crop at harvest. The opinion that potash hastens maturity and lowers the quality of the crop has not been borne out in this work.

Later Sayre and his associates (*23*) made exhaustive chemical and physical examinations of peas grown under a great variety of fertilizer treatments in the field and in water cultures. They devoted special attention to the effect of potash upon rate of maturity and quality of peas. In their conclusions it is stated: "In spite of this wide variety of conditions, only minor differences in quality of peas *of the same stage of growth* have been obtained." They further conclude: "Thus far, the belief that potash has a harmful effect upon the quality of the crop appears to be ill-founded."

Earlier work of one of the present writers (*4*) indicated the desirability of determining by more accurate methods than had previously been used the effect of potash and other fertilizer constituents upon the development of the pea as indicated by its chemical composition. Nitrogen fertilizers appeared to be of special interest, since earlier observations had suggested that a generous use of available nitrogen tends to delay maturity and to improve the quality of the pea. Accordingly, studies were made to determine the effect of certain fertilizers and of the stage of development on the composition and yield of peas.

GROWING AND HANDLING EXPERIMENTAL MATERIAL

FIELD-PLOT METHODS

The Alaska variety of pea (*Pisum sativum* L.) was used in these studies. Work was carried on at the Arlington Experiment Farm,

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Rosslyn, Va., in 1930 and 1931. All treatments were in duplicate, and each consisted of an application of a single fertilizer constituent. The treatments and rates of application per acre were as follows:

> N.—Nitrate of soda 400 pounds and sulphate of ammonia 280 pounds (about 116 pounds of nitrogen).
> P.—Superphosphate 1,000 pounds (about 160 pounds of phosphoric acid).
> K.—Muriate of potash 300 pounds (about 144 pounds of potash).
> C.—Check; no treatment.

The quantities of fertilizers used per acre were in excess of those employed in growing peas for the cannery, the object being to produce measurable differences in the composition of the products. In order to overcome as far as possible any definite gradient in soil conditions that might exist, the eight plots were arranged as follows:

Potash	Check
Phosphorus	Nitrogen
Check	Phosphorus
Nitrogen	Potash

The fertilizing materials were applied by hand after the soil had been plowed and disked once. They were then worked in by further disking and harrowing, care being taken to avoid dragging soil from one plot to another. The peas were sown with a garden drill at a depth of 1 to 1½ inches.

In 1930 the plots were located on a moderately fertile area of artificial land which had been dredged from the Potomac River and which was apparently of a silt-loam character. Each plot was 53 by 33 feet. The rows were 14 inches apart, sown at the rate of approximately 4 bushels of seed per acre. A border of 1 row along the sides and a width of 1 foot at the ends of each plot was left unharvested. In 1931 the same plots and treatments were used as in 1930. One tier of 4 plots was sown in rows 7 inches apart, at the rate of 4 bushels of seed per acre; the other tier was sown in 14-inch rows, at the rate of 2 bushels per acre. In 1930 the fertilizers were applied and seed sown on March 18, and the peas were harvested on May 28 and on June 2. In 1931 the plots were fertilized and sown on April 10 and the peas harvested on June 6 and June 10.

HARVESTING, GRADING, AND DRYING

In both 1930 and 1931 a power-driven pea huller, a hand-driven grader, and a specially constructed drier not only permitted the use of large and adequate samples but also very greatly reduced the time from harvest to complete dryness of the material.

The drier consisted of an asbestos-board cabinet 6 feet high, 4½ feet wide, and 2½ feet deep, inside measurement, with the entire front made of double doors which opened at the center. The outside was covered with a one half inch layer of builders' insulating material. Ten shallow trays 4 feet long and 2½ feet wide rested upon horizontal iron-rod supports. The bottoms of the trays were of 8-mesh hardware cloth. Each tray was supported by three three-eighth inch rods

which prevented the sagging of the center of the large screen-wire bottom. There was a 3-inch "head space" between trays. The opposite ends of alternate trays were placed against the side walls when the drier was in operation, thus forcing the hot air to travel over each tray that carried material to be dried.

Immediately to one side of the cabinet and connected with it by an opening near the base were two low-form, five-column, common steam radiators each of 50 square feet radiation. Over these radiators, which were enclosed in an asbestos-board housing, a strong current of air was forced by a centrifugal blower of a capacity of 1,500 cubic feet per minute driven by a three-horsepower electric motor. With a steam pressure of 15 pounds per square inch in the radiators, a temperature of 60° to 65° C. could be maintained in the cabinet during the drying of a considerable mass of plant material. In the brief period of 3 to 4 hours 25 to 30 pounds of peas were dried to such a degree that they rattled when handled. The material to be dried had to be spread very thinly and uniformly on the trays and moved carefully about from time to time to obtain satisfactory results.

At each harvest date the peas from one half of each plot were gathered; all pods were removed from all plants scheduled for harvest on that date. The duplicate or second series of plots was harvested in reverse order to the first, since all plots could not be harvested simultaneously. In most instances the samples of shelled peas were placed in the drier within 3 to 4 hours after the pods were harvested.

FIELD RESULTS

YIELD

The yield data are of secondary importance, since the treatments were made for a specific purpose other than for increasing yields and were of a character that ordinarily would not be recommended in field practice. However, the yield data, presented in table 1, indicate the growth relationships due to the various treatments and the general level of nutritional conditions under which the studies were made. Individual plots of the duplicate treatments are designated A and B.

Even though small differences appear between the total yields of fresh shelled peas from the various treatments in 1930 and 1931, they are of no significance, for the differences between duplicates are in general greater than between treatments. The absence of material differences in yield is not what one might expect but possibly may be accounted for in part by (1) the high fertility of the soil to which treatments were applied, and (2) by injury from excessive amounts of readily soluble nutrient salts. The latter suggestion carries little weight because no definitely injurious effects were noted except in the nitrogen-treated plots of 1930. Furthermore, in market-garden areas peas are heavily fertilized without injury. A late frost in 1930 injured the nitrogen-treated plots rather severely, but the others were not appreciably harmed. Further evidence of the lack of fertilizer injury was afforded by yields of similar plots in 1931. Germination of the pea is known to be adversely affected by excessive concentrations of fertilizer salts, but no damage was noted in any of these plots.

TABLE 1.— *Yield per acre of shelled peas (fresh weight) as affected by potash, phosphoric acid, and nitrogen in 1930 and 1931*

1930 CROP

Harvest and grade no.	Plot	Yield from treatment indicated			
		Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:		*Pounds*	*Pounds*	*Pounds*	*Pounds*
1	A	98	158	167	51
	B	118	112	179	135
2	A	182	146	218	79
	B	236	292	250	109
3	A	149	58	195	103
	B	136	374	213	73
4	A	39	--------	48	19
	B	30	68	41	31
5	A	--------	--------	12	24
	B				
All grades	A	468	362	640	276
	B	520	846	683	348
Average, A and B		494	604	662	312
Harvest of June 2:					
1	A	85	75	86	71
	B	75	99	74	37
2	A	185	168	144	80
	B	140	212	141	120
3	A	313	234	601	391
	B	434	352	573	155
4	A	401	154	321	196
	B	185	328	567	173
5	A	145	80	68	--------
	B	112	136	--------	
All grades	A	1,129	711	1,200	738
	B	946	1,127	1,355	485
Average, A and B		1,038	919	1,288	612

1931 CROP

Harvest and grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 6:					
1	A	104	164	215	122
	B	128	176	202	104
2	A	261	287	352	289
	B	303	512	444	303
3	A	234	157	192	265
	B	257	396	289	208
4	A	63	22	35	99
	B	40	84	42	36
All grades	A	662	630	794	775
	B	728	1,168	977	651
Average, A and B		695	899	886	713
Harvest of June 10:					
1	A	127	137	198	105
	B	320	212	351	260
2	A	402	382	414	316
	B	761	646	928	626
3	A	678	558	934	592
	B	943	1,166	994	882
4	A	542	462	724	580
	B	383	700	352	358
5	A	17	19	57	28
	B	--------	22	8	12
All grades	A	1,766	1,558	2,327	1,621
	B	2,407	2,746	2,633	2,138
Average, A and B		2,087	2,152	2,480	1,880

In 1930 phosphoric acid produced a definite increase in yield. In 1931 the same plot with the same phosphorus treatment also showed an increase of appreciable magnitude (table 1).

It should be borne in mind that these increases in yield were obtained on a soil dredged from the river. In past years it had received moderate applications of manure and green manure and may have been relatively low in phosphorus. Other field experiments in nearby Maryland have not shown phosphorus to be markedly effective in increasing the yield of peas. As previously explained, the low yield of the nitrogen plot in 1930 is believed to have been due to frost injury while the peas were in an early stage of growth. In 1931 nitrogen and potash applications resulted in no significant differences in yield.

MATURITY

In this investigation the possible effect of nutrients upon rate of development and properties of the peas is of more interest than the effect upon yield.

In this work the rate of development or stage of maturity has been judged on the basis of distribution of sizes of peas in the pods. This is the basis used by canners' field men in determining when a field has attained the proper stage for harvesting. In order to reduce the expression of maturity to a single numerical value, the percentage of the yield of a plot constituted by each grade was multiplied by an arbitrary weighting, for convenience, equal to the standard grade number. The sum of these products of a single harvest is designated as a "maturity index" (4). Sayre and his associates (23) have suggested, since the present work was begun, that as an index to quality this maturity index is less reliable than their "quality index."

The latter is calculated by summing the products obtained in multiplying the percentage of yield constituting each grade by the numerical value of the crushing test for that grade. However, since stage of development is commonly judged by distribution of sizes, and since this work was designed to study the pea as generally handled in field practice, the above-described maturity index is believed to be better adapted to the present investigation.

Table 2 shows the distribution of sizes of shelled peas of each harvest from each plot as well as the maturity indices for each plot and for the total yield of duplicate plots of each treatment. No appreciable consistent differences in maturity resulted from the treatments. The same was true when adjacent different treatments were compared in the field. In one comparison the difference was in favor of one plot, and, more often than not, the relationship was reversed in the second comparison. When the indices for the total plot area of each treatment are compared, they are found to be quite similar for any one harvest date.

There was no evident difference in the time of blossoming of the plants in any of the several plots. Thus, earlier observations in the field, and the reports of other investigators (4, 23), appear to be confirmed under additional and quite different conditions as reported in this paper.

TABLE 2.—*Influence of potash, phosphoric acid, and nitrogen upon stage of maturity of peas at harvest in 1930 and 1931*

1930 CROP

Harvest and grade no.	Plot	Yield from treatment indicated			
		Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:		*Percent*	*Percent*	*Percent*	*Percent*
1 and smaller	{A	20. 98	43. 70	26. 01	18. 37
	{B	22. 70	13. 20	26. 20	38. 80
2	{A	38. 88	40. 35	34. 11	28. 55
	{B	45. 29	34. 48	36. 60	31. 40
3	{A	31. 79	15. 93	30. 50	37. 52
	{B	26. 20	44. 24	31. 20	21. 06
4	{A	8. 36	----------	7. 48	6. 98
	{B	5. 80	8. 07	5. 97	8. 74
5	{A	----------	----------	1. 89	8. 58
	{B				
Maturity index	{A	228	172	216	259
	{B	215	247	217	200
Maturity index of total A and B		221	225	221	226
Harvest of June 2:					
1 and smaller	{A	7. 50	10. 54	7. 04	9. 63
	{B	7. 99	8. 78	5. 44	7. 61
2	{A	16. 42	23. 63	11. 78	10. 81
	{B	14. 80	18. 81	10. 43	24. 75
3	{A	27. 72	32. 92	49. 35	53. 00
	{B	45. 85	31. 20	42. 27	31. 90
4	{A	35. 53	21. 69	26. 30	26. 56
	{B	19. 57	29. 08	41. 85	35. 75
5	{A	12. 81	11. 23	5. 55	----------
	{B	11. 83	12. 09	----------	----------
Maturity index	{A	330	300	312	293
	{B	313	317	321	296
Maturity index of total A and B		322	310	316	296

1931 CROP

Harvest of June 6:					
1	{A	15. 71	26. 02	27. 08	15. 74
	{B	17. 59	15. 08	20. 67	15. 97
2	{A	39. 42	45. 56	44. 33	37. 30
	{B	41. 60	43. 82	45. 44	46. 55
3	{A	35. 36	24. 92	24. 19	34. 20
	{B	35. 30	33. 90	29. 51	31. 94
4	{A	9. 52	3. 49	4. 41	12. 76
	{B	5. 49	7. 19	4. 30	5. 53
Maturity index	{A	239	206	206	244
	{B	229	233	217	227
Maturity index of total A and B		233	224	212	236
Harvest of June 10:					
1	{A	7. 19	8. 79	8. 52	6. 48
	{B	13. 30	7. 72	13. 33	12. 15
2	{A	22. 75	24. 51	17. 82	19. 49
	{B	31. 61	23. 52	35. 23	29. 28
3	{A	38. 40	35. 82	40. 05	36. 51
	{B	39. 20	42. 48	37. 72	41. 26
4	{A	30. 69	29. 67	31. 15	35. 78
	{B	15. 90	25. 49	13. 37	16. 74
5	{A	. 96	1. 20	2. 45	1. 73
	{B	. 00	. 80	. 34	. 59
Maturity index	{A	295	290	301	307
	{B	258	288	252	264
Maturity index of total A and B		274	289	275	283

PHYSIOLOGICAL STUDIES

Chemical analyses in connection with studies similar to those herein recorded have often been reported and discussed on the basis of oven-dry matter. Such a method of presentation has its proper applications, but alone would not be suitable here for the following reasons: (1) The study was designed to show the development and composition of fresh peas in response to certain field practices, therefore the results must be considered as they apply to fresh material; (2) peas grown in the garden or for the cannery are consumed on the fresh-weight basis, i.e., in the moisture-containing condition, so their composition as affecting nutritional value should be expressed on that basis; and (3) the results of this work when expressed on the dry-weight basis lead to conclusions for the most part opposite to, or quite different from, those reached when analyses are expressed on the fresh-weight basis. Consequently, analytical data are presented on both a fresh- and a dry-weight basis, but are discussed mainly on the fresh-weight basis. Certain facts are revealed which heretofore have not been plainly evident from the work of others who have reported their results on the basis of oven-dry matter only.

DRY MATTER

After the peas had been harvested, weighed, shelled, and graded, they were subjected to preliminary drying at 60° to 65° C. for 24 hours in the specially designed forced-draft drying apparatus already described. After drying, the samples were left for a few days in a dry room with access to the air, before they were weighed. This was done to reduce errors that might arise from the absorption of moisture during subsequent handling and grinding. The samples were ground to pass a 40-mesh sieve, and stored in tightly stoppered bottles until analyzed. The moisture content of the air-dry samples that had been dried in a preliminary way in the forced-draft apparatus was determined by drying them in an electric oven at a temperature of 103° to 105°, until constant weight was obtained. The results are recorded in table 3.

Table 3 shows that the percentage differences in the dry-matter content of pea samples grown in differently treated plots are insignificant, being mostly smaller than the percentage differences between samples from duplicate plots. For instance, the mean dry-matter percentages of grade 2, 3, and 4 peas from the untreated plot shown by samples 1, 3, and 5 and their duplicate samples 2, 4, and 6 are 22.37 and 22.87, respectively, making a difference of 0.50 percent. On the other hand, the difference between the dry-matter content of samples 2, 4, and 6 from the untreated plot (22.87) and from the potash-treated plot (23.14) is only 0.27 percent.

TABLE 3.—*Percentage of oven-dry matter in the Alaska pea as affected by different fertilizers; determined on the basis of fresh weight, 1930 and 1931*

1930 CROP

Harvest and sample no.	Grade no.	Plot	Dry matter from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	20.46	21.06	20.73	20.68
2	2	B	21.03	21.05	21.34	21.03
		Average	20.74	21.05	21.03	20.85
3	3	A	22.73	23.56	22.30	21.74
4	3	B	23.48	23.21	23.34	22.84
		Average	23.10	23.38	22.82	22.29
5	4	A	23.92	....	23.97	22.78
6	4	B	24.10	25.16	25.19	23.63
		Average	24.01	25.16	24.58	23.20
		Average (1–6)	22.62	22.81	22.81	22.11
Harvest of June 2:						
7	2	A	23.78	24.48	25.17	22.81
8	2	B	24.90	24.44	25.69	24.84
		Average	24.34	24.46	25.43	23.82
9	3	A	27.70	27.76	27.88	25.25
10	3	B	28.45	28.03	29.08	28.47
		Average	28.07	27.89	28.48	26.86
11	4	A	29.41	29.53	30.27	29.33
12	4	B	31.17	29.92	31.39	29.76
		Average	30.29	29.72	30.83	29.54
		Average (7–12)	27.57	27.36	28.25	26.74
		Average (1–12)	25.09	25.30	25.53	24.43

1931 CROP

Harvest of June 10:						
13	2	A	20.15	20.08	....	20.94
14	2	B	21.01	21.20	....	20.96
		Average	20.58	20.64	....	20.95
15	3	A	24.89	24.81	....	25.57
16	3	B	25.30	26.05	....	25.21
		Average	25.10	25.43	....	25.39
17	4	A	28.57	28.11	....	28.83
18	4	B	27.64	28.66	....	29.37
		Average	28.11	28.39	....	29.10
		Average (13–18)	24.59	24.82	....	25.15

ASH CONTENT

The ashing of the pea samples was effected in an electric muffle at a dull red heat, until constant weight was obtained. The ash was white and fluffy. In no case was trickling allowed to occur. The percentages of ash in the various pea samples are recorded in table 4. In view of the importance of the ash content in foodstuffs (5), the following regularities are pointed out.

The ash content was greater in the riper large-sized peas than in the unripe small-sized peas from both the 1930 and 1931 crops, although it did not increase in proportion to the dry matter. This

was true of all samples from a single plot at one harvest with but one exception. Another regular trend revealed by the ash results is that without exception peas of the same grade from the same plot but harvested later had a larger ash content than those harvested earlier. This increase in ash in the later harvested peas is most closely associated with increase in total dry matter; however, on the dry-weight basis the ash content ordinarily decreases with the size and age of the peas. It seems reasonable to ascribe this decrease to the fact that as the growth of the peas progresses the proportions of organic reserve substances such as carbohydrates and proteins increase more rapidly than the proportions of ash from the soil.

TABLE 4.—*Percentage of total ash in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and oven-dry weight, 1930 and 1931*

1930 CROP, FRESH WEIGHT

Harvest and sample no.	Grade no.	Plot	Ash from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	0.771	0.811	0.771	0.781
2	2	B	.751	.816	.785	.776
		Average	.761	.813	.778	.778
3	3	A	.816	.841	.800	.787
4	3	B	.794	.850	.822	.827
		Average	.805	.845	.811	.807
5	4	A	.854	....	.851	.793
6	4	B	.816	.900	.881	.827
		Average	.835	.900	.866	.810
		Average (1–6)	.800	.844	.818	.798
Harvest of June 2:						
7	2	A	.855	.862	.825	.840
8	2	B	.859	.932	.860	.832
		Average	.857	.897	.842	.836
9	3	A	.942	.922	.911	.883
10	3	B	.934	1.026	.922	.922
		Average	.938	.974	.916	.902
11	4	A	.982	.978	.962	.997
12	4	B	.992	1.069	.986	.955
		Average	.987	1.023	.974	.976
		Average (7–12)	.927	.965	.911	.905
		Average (1–12)	.863	.910	.864	.851

1931 CROP, FRESH WEIGHT

Harvest and sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	0.915	0.915	....	0.923
14	2	B	.907	.906	....	.959
		Average	.911	.911	....	.941
15	3	A	1.015	.987	....	1.026
16	3	B	.986	.978	....	1.024
		Average	1.001	.983	....	1.025
17	4	A	1.110	1.074	....	1.167
18	4	B	1.039	1.047	....	1.080
		Average	1.075	1.061	....	1.124
		Average (13–18)	.995	.985	....	1.030

TABLE 4.—*Percentage of total ash in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*—Contd.

1930 CROP, OVENDRY WEIGHT

Harvest and sample no.	Grade no.	Plot no.	Ash from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	3.77	3.85	3.72	3.78
2	2	B	3.57	3.88	3.68	3.69
		Average	3.67	3.87	3.70	3.74
3	3	A	3.59	3.57	3.59	3.62
4	3	B	3.38	3.66	3.52	3.62
		Average	3.49	3.62	3.56	3.62
5	4	A	3.57		3.55	3.48
6	4	B	3.37	3.58	3.50	3.50
		Average	3.47	3.58	3.53	3.49
		Average (1–6)	3.54	3.71	3.59	3.62
Harvest of June 2:						
7	2	A	3.60	3.52	3.28	3.68
8	2	B	3.45	3.81	3.35	3.35
		Average	3.53	3.67	3.32	3.52
9	3	A	3.40	3.32	3.27	3.50
10	3	B	3.28	3.66	3.17	3.24
		Average	3.34	3.49	3.22	3.37
11	4	A	3.34	3.31	3.18	3.40
12	4	B	3.18	3.57	3.14	3.21
		Average	3.26	3.44	3.16	3.31
		Average (7–12)	3.38	3.53	3.23	3.40
		Average (1–12)	3.46	3.61	3.41	3.51

1931 CROP, OVEN-DRY WEIGHT

Harvest of June 10:						
13	2	A	4.27	4.21		4.13
14	2	B	4.06	4.02		4.32
		Average	4.17	4.12		4.23
15	3	A	3.81	3.75		3.80
16	3	B	3.69	3.57		3.88
		Average	3.75	3.66		3.84
17	4	A	3.69	3.61		8.82
18	4	B	3.54	3.46		3.53
		Average	3.62	3.54		3.68
		Average (13–18)	3.84	3.77		3.91

The difference between the ash content of peas grown in fertilized and unfertilized soil is rather insignificant, in most instances being about equal to, or even less than, the difference in the ash content of peas grown in duplicate plots. The differences in mean ash content of the phosphorus- or nitrogen-treated lots as compared with the untreated ones are certainly insignificant. It appears, however, that the potash-treated plots harvested May 28, 1930, showed a significant but small increase over the checks. In the harvests of June 2, 1930, and June 10, 1931, when the peas were at the stage usually harvested for the cannery, this difference was less striking and, indeed, was of

doubtful consequence. The mean ash content of the samples from the phosphorus- and nitrogen-treated plots of this latter harvest are in remarkably close agreement with the check.

For the qualitative examination of the ash a few grams of the peas, grade 3, which were grown in soil not treated with fertilizer (table 4, no. 3, fresh-weight basis) were ashed in an electric muffle oven. The ash obtained was partly insoluble in cold and hot water but dissolved readily with the addition of a few drops of hydrochloric, nitric, sulphuric, or phosphoric acid. The ash was found to contain the following elements: Aluminum (large amount); iron, both ferric and ferrous (very little); calcium, magnesium, potassium (large amount); sodium (trace); the acids sulphuric, phosphoric (large amount), and hydrochloric (trace).

ETHER EXTRACT

Ordinarily 5- or 10-g portions of the finely ground peas were dried in an oven at 100° C. for 1 hour, after which they were transferred to fat-free paper thimbles and covered with fat-free cotton. The thimbles were than placed in the Soxhlet extraction apparatus, in which the substance was extracted with anhydrous ether for 5 hours; the ether was then driven off, the residue in the previously weighed extraction flask dried at 100° for 1 to 2 hours, cooled, and weighed.

TABLE 5.—*Percentage of ether-soluble substances in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight*

1930 CROP, FRESH WEIGHT

| Sample no. | Grade no. | Plot | Ether-soluble substances from treatment indicated | | | |
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	0.344	0.411	0.346	0.372
2	2	B	.318	.440	.414	.385
		Average	.331	.425	.380	.378
3	3	A	.382	.396	.370	.348
4	3	B	.385	.427	.387	.375
		Average	.383	.411	.378	.361
5	4	A	.335		.347	.369
6	4	B	.349	.433	.347	.359
		Average	.342	.433	.347	.364
		Average (1-6)	.352	.421	.368	.368
Harvest of June 2:						
7	2	A	.378	.392	.373	.383
8	2	B	.428	.411	.383	.405
		Average	.403	.401	.378	.394
9	3	A	.421	.419	.398	.381
10	3	B	.435	.460	.419	.407
		Average	.428	.439	.408	.394
11	4	A	.424	.446	.412	.423
12	4	B	.436	.464	.424	.432
		Average	.430	.455	.418	.427
		Average (7-12)	.420	.432	.401	.405
		Average (1-12)	.386	.427	.384	.386

TABLE 5.—*Percentage of ether-soluble substances in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight*—Con.

1931 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Ether-soluble substances from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:			*Percent*	*Percent*	*Percent*	*Percent*
13	2	A	0.388	0.361	----------	0.346
14	2	B	.395	.368	----------	.333
		Average	.392	.365	----------	.340
15	3	A	.469	.410	----------	.405
16	3	B	.433	.392	----------	.375
		Average	.451	.401	----------	.390
17	4	A	.493	.464	----------	.458
18	4	B	.476	.472	----------	.459
		Average	.485	.468	----------	.459
		Average (13–18)	.442	.411	----------	.396

1930 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:						
1	2	A	1.68	1.95	1.67	1.80
2	2	B	1.51	2.09	1.94	1.83
		Average	1.60	2.02	1.81	1.82
3	3	A	1.68	1.68	1.66	1.60
4	3	B	1.64	1.84	1.66	1.64
		Average	1.66	1.76	1.66	1.62
5	4	A	1.40	----------	1.45	1.62
6	4	B	1.45	1.72	1.38	1.52
		Average	1.43	1.72	1.42	1.57
		Average (1–6)	1.56	1.86	1.63	1.67
Harvest of June 2:						
7	2	A	1.59	1.60	1.48	1.68
8	2	B	1.72	1.68	1.49	1.63
		Average	1.66	1.64	1.49	1.66
9	3	A	1.52	1.49	1.43	1.51
10	3	B	1.53	1.64	1.44	1.43
		Average	1.53	1.57	1.44	1.47
11	4	A	1.44	1.51	1.36	1.44
12	4	B	1.40	1.55	1.35	1.45
		Average	1.42	1.53	1.36	1.45
		Average (7–12)	1.53	1.58	1.43	1.52
		Average (1–12)	1.55	1.70	1.53	1.60

1931 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	1.81	1.66	----------	1.55
14	2	B	1.77	1.63	----------	1.50
		Average	1.79	1.65	----------	1.53
15	3	A	1.76	1.56	----------	1.50
16	3	B	1.62	1.43	----------	1.42
		Average	1.69	1.50	----------	1.46
17	4	A	1.64	1.56	----------	1.50
18	4	B	1.62	1.56	----------	1.50
		Average	1.63	1.56	----------	1.50
		Average (13–18)	1.70	1.57	----------	1.50

By reference to table 5 it will be seen that, in the early stages of development, the percentages of the fat (ether extract) determined on the fresh-weight basis show no consistent relation to the grades (sizes) of the pea samples.   This is probably due to differences in maturity of peas even of the same size in different plots.   In the harvest of June 2, 1930, the larger sizes show, with one exception, a higher fat content than the next lower size.   The same is true of the harvest of June 10, 1931.   These differences are often quite small and of doubtful importance, but they are consistent.

It is evident from table 5 that peas of the same grade but harvested at a later date have, in general, a higher fat content than peas harvested earlier.   Generally speaking, the percentages of ether extract parallel the percentages of ash, this being true not only of the grades but also of the age of the corresponding pea samples.

For reasons not known the fat content of the samples of grade 2 peas from the duplicate potash plot (samples 1 and 2) and the sample of one phosphorus plot (sample 2) were higher for the May 28 harvest than for the June 2 harvest.   However, these 3 exceptions among 23 comparisons should not invalidate the generalization just drawn.

If the percentages of ether extract are calculated upon a dry-weight basis, all the above relationships are generally reversed, as table 5 shows.

The mean fat content of the three grades of peas from the untreated, the phosphorus-treated, and the nitrogen-treated plots for 1930 are remarkably similar for each harvest and for the two harvests combined.   Appreciable differences occur between corresponding samples, but they are somewhat inconsistent.   The fat content of the samples from the potash-treated plots was higher than the others in 9 out of 11 instances in 1930, but in 1931 it was lower in all 6 instances than the samples from the untreated plots, resulting in no significant [3] difference for the 2 years.   In 1931 the fat content of the nitrogen-treated plots was significantly lower than the check and potash-treated plots, but the differences were quite small and unimportant.   Although of doubtful consequence from a practical or culinary standpoint, this fact is interesting in a way that will be discussed later.   Upon a dry-weight basis these differences are less striking than when considered as percentages of fresh weight.   In 1931 the relationships just discussed for 1930 did not hold.   Thus it is not possible to attach any importance to the higher fat content of the potash plots in 1930.

In practically all cases the ether extracts were found to contain not only fat but also pigments, free fatty acids, lecithin, and apparently other constituents as well.   The following facts substantiate this statement.   All the ether extracts were more or less colored, the color ranging from very light green or light yellow to dark yellowish green.   As a rule the ether extract, at least the greater portion of it, was readily soluble in alcohol, even at room temperature.   Blue litmus paper dipped into such an alcoholic solution did not change color, but when it was subsequently wet with water the color immediately changed to red.   The same reaction is shown by the isolated higher fatty acids.   A few crystals of chemically pure palmitic or stearic acid were dissolved in alcohol and blue litmus paper was immersed in the solution.   No change of color took place; but if the litmus

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[3] As used in the discussion of results in this paper, the term "significant" or "significance" refers to differences between means for which "odds of significance" are greater than 35 to 1, by the method of Student (*1*)

paper was then dipped in water, the blue color changed to red. This reaction, showing the presence of free fatty acids, was obtained with all the extracts of the various grades of peas.

Several ether extracts of the Alaska pea, after being weighed, were taken up with ether, and the ether evaporated on the water bath. The residue was boiled with barium hydroxide for about 2 hours, after which the barium salts of the fatty acids were filtered off and the filtrate evaporated on the water bath at low temperature. The residue was taken up with warm absolute alcohol, in order to remove any choline present, and the whole filtered. The residue on the filter showed the following properties: It was soluble in water, insoluble in absolute alcohol; it contained some barium and, on oxidation with a mixture of potassium nitrate and sodium carbonate, gave a slight reaction for phosphoric acid. These facts point to the presence of lecithin in the ether extracts.

While the ether extracts of the various pea samples were found to contain, in addition to glycerides, also pigments, free fatty acids, and lecithin, they differed from one another both quantitatively and qualitatively. In other words, the pea samples contained not only unequal proportions of ether extracts but they differed also in shade and intensity of color, strength of acidity, solubility, etc. For instance, unlike most of the ether extracts, two of them solidified, at least in part, and were with difficulty soluble in alcohol. The solidified substance may have consisted of wax or a waxlike matter whose nature, however, was not studied. The occurrence of waxlike substances in peas has previously been reported by Schulze and his collaborators (*25*). It is not unreasonable to assume that a careful study of the various constituents of the ether extract may throw additional light on the quality of the Alaska pea.

CARBOHYDRATES

Two grams of ground peas was transferred to a paper extraction thimble which was then covered with glass wool to prevent the substance from being thrown out of the thimble and to insure good uniform extraction. This was effected in a Soxhlet extraction apparatus by means of 60-percent alcohol, which was added to the extraction flask in a quantity sufficient to fill the extractor to the top of the siphon (usually 220 cc), leaving at the same time enough alcohol in the flask to prevent caramelization of the sugars by heat incidental to the extraction. Extraction was continued for at least 4 hours. At the expiration of this time the extract was, with the aid of hot water, transferred quantitatively to an evaporating dish and the alcohol driven off on the water bath. The alcohol-free extract was then transferred by means of hot water to a 250-cc volumetric flask, about 1 cc of 20-percent neutral lead acetate solution added for clarification, made up to the mark, and filtered until perfectly clear.

ESTIMATION OF REDUCING SUGARS

The method employed for the estimation of reducing sugars was that of Bertrand (*2, 8, 9, 21, 22*). Ordinarily a 100-cc portion of the clear filtrate was taken for this estimation. The solution was transferred to an Erlenmeyer flask of about 200- to 250-cc capacity, 20 cc of Bertrand's solution A (copper sulphate) and 20 cc of Bertrand's solution B (Rochelle salt) added, the whole brought to a boil, and

the boiling continued for 3 minutes. The flask was then removed from the flame and the cuprous oxide precipitate allowed to settle well, when the supernatant blue liquid was decanted through a Soxhlet asbestos filter and sucked off. The cuprous oxide precipitate, both in the flask and on the filter, was then washed repeatedly with hot distilled water, after which the filter was removed from the suction flask, and the latter was carefully washed with water to remove any copper present. About 20 cc of Bertrand's solution C (ferric sulphate) was placed in the Erlenmeyer flask, and this dissolved all the cuprous oxide present. This solution was then poured on the Soxhlet filter, placed on the suction flask, and sucked through slowly to dissolve any cuprous oxide present on the filter. The flask was then washed with water, the wash water being used also to wash the Soxhlet filter in order to get quantitatively all the copper solution into the suction flask. This solution was then titrated with Bertrand's solution D (standard potassium permanganate) to a pink color. The results were calculated as glucose.

The potassium permanganate solution was standardized against ammonium oxalate.

DETERMINATION OF TOTAL SUGARS

For the determination of total sugars 75 or 100 cc of the solution, as employed for the estimation of reducing sugars, was used. The inversion was effected by the method of Herzfeld (*7, 10*). The solution was transferred to a 100-cc volumetric flask, 8 cc of 36-percent hydrochloric acid was added, and the whole shaken carefully. The flask with its contents was heated to 67° to 70° C. by placing it up to the neck in a water bath at 70°, this temperature being maintained for 5 more minutes. Total inversion was never allowed to last more than 10 minutes. At the expiration of that time the flask was cooled with cold water, the hydrolysate neutralized with sodium hydroxide solution and made up to 100 cc. This was divided into two equal portions and employed for the determination of total sugars according to Bertrand's method as already outlined. The results were calculated as invert sugar.

ESTIMATION OF SUCROSE

Sucrose was calculated by subtracting the percentage of reducing sugars from that of total sugars and multiplying the difference by the factor 0.95.

ESTIMATION OF STARCH

Strictly speaking, the estimation of starch deals with total acid-hydrolyzable substances rather than with starch alone, since the pea is known to contain, in addition to starch, other acid-hydrolyzable polysaccharides such as sucrose, cellulose, dextrin, hemicelluloses (xylan, araban, galactan, and mannan), and perhaps other polysaccharides and substances that yield some glucose upon hydrolysis. In this connection the following facts should be borne in mind: Sucrose has been quantitatively removed from the peas by extraction with 60-percent alcohol prior to hydrolysis; cellulose is insoluble in 2-percent hydrochloric acid used for hydrolysis of the polysaccharides; dextrin is known to occur in the pea to the extent of but 6 percent and may have been wholly or partly extracted by the 60-percent

alcohol, while the exact nature and quantities of all hemicelluloses occurring in peas are not very well known. It cannot be doubted that the hemicelluloses are hydrolyzed, at least in part, under the conditions of hydrolysis as employed in this investigation. However, it seems fairly safe to state that the changes of total hydrolyzable substances give a good index to the changes in starch content. For this reason the acid-hydrolyzable polysaccharides are referred to here as starch.

The starch was estimated essentially according to the method described by Lohrisch (*17, p. 375*), Zemplén (*27*), and Schmidt (*24, p. 924*). The residue that remained from the 2 g of peas, after the sugars had been extracted with 60-percent alcohol, was transferred to a 1-l [4] flask, to which 150 cc of distilled water and 8 cc of 36-percent hydrochloric acid were added, and the mixture was hydrolyzed by boiling gently for 2 hours. The hydrolysate was then cooled, neutralized with sodium hydroxide solution, transferred to a 250-cc volumetric flask, made up to volume with water, and filtered clear. Ten-cubic-centimeter portions of this solution were used for estimating the sugar as glucose according to Bertrand's method. The starch was calculated by multiplying the glucose found by the factor 0.9. The results obtained with the various carbohydrates are summarized in tables 6, 7, and 8.

TABLE 6.—*Percentage of reducing sugars in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*

1930 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Reducing sugars from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	0.039	0.048	0.039	0.079
2	2	B	.034	.042	.041	.080
		Average	.036	.045	.040	.079
3	3	A	.064	.045	.033	.061
4	3	B	.045	.070	.037	.075
		Average	.054	.057	.035	.068
5	4	A	.048		.031	.064
6	4	B	.036	.058	.030	.054
		Average	.042	.058	.030	.059
		Average (1–6)	.044	.053	.035	.069
Harvest of June 2:						
7	2	A	.071	.056	.048	.082
8	2	B	.029	.037	.044	.060
		Average	.050	.046	.046	.071
9	3	A	.042	.033	.078	.096
10	3	B	.037	.081	.047	.088
		Average	.039	.057	.062	.092
11	4	A	.050	.050	.048	.100
12	4	B	.034	.093	.041	.048
		Average	.042	.071	.044	.074
		Average (7–12)	.043	.058	.051	.079
		Average (1–12)	.044	.056	.043	.074

[4] A large flask is indispensable, otherwise loss of liquid may occur at the beginning of boiling, when the liquid foams quite badly.

TABLE 6.—*Percentage of reducing sugars in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*—Continued

1931 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Reducing sugars from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:			*Percent*	*Percent*	*Percent*	*Percent*
13	2	A	0.036	0.043		0.074
14	2	B	.042	.034		.084
		Average	.039	.039		.073
15	3	A	.040	.039		.057
16	3	B	.053	.060		.053
		Average	.047	.050		.055
17	4	A	.042	.059		.113
18	4	B	.059	.061		.040
		Average	.051	.060		.077
		Average (13–18)	.045	.049		.070

1930 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:						
1	2	A	0.19	0.23	0.19	0.38
2	2	B	.16	.20	.19	.38
		Average	.18	.22	.19	.38
3	3	A	.28	.19	.15	.28
4	3	B	.19	.30	.16	.33
		Average	.24	.25	.16	.31
5	4	A	.20		.13	.28
6	4	B	.15	.23	.12	.23
		Average	.18	.23	.13	.26
		Average (1–6)	.20	.23	.16	.31
Harvest of June 2:						
7	2	A	.30	.23	.19	.36
8	2	B	.12	.15	.17	.24
		Average	.21	.19	.18	.30
9	3	A	.15	.12	.28	.38
10	3	B	.13	.29	.16	.31
		Average	.14	.21	.22	.35
11	4	A	.17	.17	.16	.34
12	4	B	.11	.31	.13	.16
		Average	.14	.24	.15	.25
		Average (7–12)	.16	.21	.18	.30
		Average (1–12)	.18	.22	.17	.31

1931 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	0.17	0.20		0.33
14	2	B	.19	.15		.38
		Average	.18	.18		.36
15	3	A	.15	.15		.21
16	3	B	.20	.22		.20
		Average	.18	.19		.21
17	4	A	.14	.20		.37
18	4	B	.20	.20		.13
		Average	.17	.20		.25
		Average (13–18)	.18	.19		.27

TABLE 7.—*Percentage of sucrose in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*

1930 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Sucrose from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	4.22	3.62	3.98	4.59
2	2	B	3.55	3.61	3.89	3.93
		Average	3.88	3.61	3.93	4.26
3	3	A	3.35	2.83	3.74	4.03
4	3	B	2.72	3.03	3.31	3.53
		Average	3.03	2.93	3.52	3.78
5	4	A	3.46	....	3.28	3.47
6	4	B	2.53	2.88	2.87	3.02
		Average	2.99	2.88	3.07	3.24
		Average (1–6)	3.30	3.19	3.51	3.76
Harvest of June 2:						
7	2	A	4.14	3.16	3.62	4.20
8	2	B	2.90	3.83	3.29	3.57
		Average	3.52	3.49	3.45	3.88
9	3	A	2.65	2.82	3.28	3.27
10	3	B	2.41	3.13	2.94	2.55
		Average	2.53	2.97	3.11	2.91
11	4	A	2.38	2.38	2.98	3.21
12	4	B	2.43	3.21	2.56	2.57
		Average	2.40	2.79	2.77	2.89
		Average (7–12)	2.82	3.09	3.11	3.23
		Average (1–12)	3.06	3.14	3.31	3.49

1931 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	3.42	4.11	....	3.86
14	2	B	3.81	3.61	....	4.23
		Average	3.62	3.86	....	4.05
15	3	A	3.08	3.08	....	2.91
16	3	B	2.77	2.98	....	3.40
		Average	2.93	3.03	....	3.16
17	4	A	2.54	2.55	....	2.22
18	4	B	2.99	2.65	....	3.45
		Average	2.77	2.60	....	2.84
		Average (13–18)	3.10	3.16	....	3.35

TABLE 7.—*Percentage of sucrose in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*—Con.

1930 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Sucrose from treatment indicated			
			Check	Potash	Phos-phoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	20.61	17.19	19.18	22.21
2	2	B	16.87	17.14	18.24	18.67
		Average	18.74	17.17	18.71	20.44
3	3	A	14.74	12.01	16.77	18.52
4	3	B	11.60	13.05	14.17	15.44
		Average	13.17	12.53	15.47	16.98
5	4	A	14.48	_____	13.69	15.23
6	4	B	10.51	11.45	11.35	14.04
		Average	12.50	11.45	12.52	14.64
		Average (1–6)	14.80	14.17	15.57	17.35
Harvest of June 2:						
7	2	A	17.39	12.81	14.37	18.43
8	2	B	11.64	15.68	12.81	14.38
		Average	14.52	14.25	13.59	16.41
9	3	A	9.56	10.18	11.76	12.94
10	3	B	8.47	11.17	10.10	8.96
		Average	9.02	10.68	10.93	10.95
11	4	A	8.10	8.07	9.85	10.94
12	4	B	7.78	10.74	8.15	8.65
		Average	7.94	9.41	9.00	9.80
		Average (7–12)	10.49	11.44	11.17	12.38
		Average (1–12)	12.65	12.68	13.37	14.87

1931 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phos-phoric acid	Nitrogen
Harvest of June 10:						
13	2	A	15.96	18.90	_____	17.28
14	2	B	17.05	16.02	_____	19.07
		Average	16.51	17.46	_____	18.18
15	3	A	11.58	11.69	_____	10.77
16	3	B	10.35	10.89	_____	12.90
		Average	10.97	11.29	_____	11.84
17	4	A	8.44	8.59	_____	7.27
18	4	B	10.20	8.77	_____	11.29
		Average	9.32	8.68	_____	9.28
		Average (13–18)	12.26	12.48	_____	13.10

TABLE 8.—*Percentage of acid-hydrolyzable substances in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*

1930 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Acid-hydrolyzable substances from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	5.82	5.07	5.69	5.00
2	2	B	6.01	6.70	6.22	6.44
		Average	5.91	5.88	5.95	5.72
3	3	A	7.66	8.06	6.90	6.60
4	3	B	8.40	8.16	8.38	7.83
		Average	8.03	8.11	7.59	7.21
5	4	A	8.38		8.03	7.81
6	4	B	8.66	9.84	8.96	8.19
		Average	8.52	9.84	8.49	8.00
		Average (1-6)	7.49	7.56	7.34	6.98
Harvest of June 2:						
7	2	A	8.24	8.31	8.77	6.93
8	2	B	8.92	8.73	8.87	8.14
		Average	8.58	8.52	8.82	7.53
9	3	A	11.07	9.56	10.60	10.10
10	3	B	11.81	11.10	12.05	11.15
		Average	11.44	10.33	11.32	10.62
11	4	A	12.34	11.85	12.48	12.30
12	4	B	13.32	13.60	13.83	11.82
		Average	12.83	12.72	13.15	12.06
		Average (7-12)	10.95	10.53	11.10	10.07
		Average (1-12)	9.22	9.18	9.22	8.52

1931 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	5.97	6.53		7.18
14	2	B	6.60	7.53		6.48
		Average	6.29	7.03		6.83
15	3	A	10.20	9.55		10.20
16	3	B	10.07	10.89		9.30
		Average	10.14	10.22		9.75
17	4	A	12.54	12.67		11.91
18	4	B	11.82	13.19		12.50
		Average	12.18	12.93		12.21
		Average (13-18)	9.53	10.06		9.60

TABLE 8.—*Percentage of acid-hydrolyzable substances in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*—Continued

1930 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Acid-hydrolyzable substances from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	28.49	24.10	27.42	24.19
2	2	B	28.59	31.82	29.13	30.48
		Average	28.54	27.96	28.28	27.34
3	3	A	33.71	34.22	30.94	30.39
4	3	B	35.79	35.17	35.93	34.30
		Average	34.75	34.70	33.44	32.35
5	4	A	35.06		33.52	34.30
6	4	B	35.91	39.13	35.60	34.65
		Average	35.49	39.13	34.56	34.48
		Average (1-6)	32.93	32.89	32.09	31.39
Harvest of June 2:						
7	2	A	34.65	33.97	34.84	30.39
8	2	B	35.82	35.71	34.55	32.77
		Average	35.24	34.84	34.70	31.58
9	3	A	39.99	34.43	38.01	40.00
10	3	B	41.51	39.60	41.45	39.18
		Average	40.75	37.02	39.73	39.59
11	4	A	41.94	40.11	41.21	41.96
12	4	B	42.74	45.45	44.07	39.71
		Average	42.34	42.78	42.64	40.84
		Average (7-12)	39.44	38.21	39.02	37.34
		Average (1-12)	36.18	35.79	35.56	34.36

1931 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	27.86	30.06		32.16
14	2	B	29.54	33.38		29.20
		Average	28.70	31.72		30.68
15	3	A	38.29	36.28		37.78
16	3	B	37.67	39.76		35.22
		Average	37.98	38.02		36.50
17	4	A	41.68	42.61		38.98
18	4	B	40.26	43.59		40.88
		Average	40.97	43.10		39.93
		Average (13-18)	35.88	37.61		35.70

DISCUSSION AND INTERPRETATION OF CARBOHYDRATE RESULTS

From table 6 it will be seen that, as a rule, the proportions of reducing sugars are almost negligible, ranging from 0.03 to 0.10 percent in the 1930 peas, and from 0.036 to 0.113 percent in the 1931 peas, calculated on the basis of fresh weight. There appears to be some difference in the percentage of reducing sugars obtained from peas grown on the variously treated plots. Thus, in the case of the 1930

peas raised in untreated soil the reducing sugars ranged from 0.029 to 0.071 percent, these extremes being duplicates; the average of all samples was 0.044 percent. Very similar results were obtained with the peas grown in phosphorus-treated soil, in which the percentage of reducing sugar ranged from 0.03 to 0.078, the average being 0.043 percent. Somewhat larger was the proportion of reducing sugars in peas from potash-treated soil, in which the percentage ranged from 0.033 to 0.093, with an average of 0.056 percent in 1930. However, the differences between corresponding samples were so inconsistent that no significance can be attached to the differences between the means just referred to. In peas from the nitrogen-treated plot the proportions of reducing sugars were slightly and significantly larger than from the check or those receiving other treatments. The analytical averages of the 1931 peas are similar to those of the 1930 peas. Considering the fact that the percentage of reducing sugars is extremely small, averaging 0.044 and 0.045 percent in peas from the untreated plot for 1930 and 1931, respectively, it would appear that there is some physiological significance in the higher percentage of reducing sugars in peas from the nitrogen-treated plot (average, 0.074 and 0.070 percent for 1930 and 1931, respectively) as compared with that in the peas from the other plots. This higher content of reducing substances, although significant statistically, is of such small magnitude as to be of no practical importance. The foregoing observations concerning determinations made on the fresh-weight basis apply equally to those made on the oven-dry weight basis.

Table 7 reveals distinct regularities in the proportions of sucrose. The figures for total sugars have been omitted, since sucrose constitutes 96 to 99 percent of the total sugars and the reducing sugars are negligible. The large-sized peas had a smaller percentage of sucrose than the small-sized peas, regardless of the time of harvest or the treatment of the plot. The older peas had a smaller sucrose content than the younger ones of the same size, age and sucrose content standing in reverse ratio.

The mean percentages of sucrose in the peas from the untreated and potash-treated plots were practically the same. The sucrose percentage in peas that received the phosphorus treatment was significantly higher than that of the check, but not significantly different from that of peas from the other plots.

The regularities in sucrose content of the peas on the fresh-weight basis hold good also for that of peas on the dry-weight basis, but the differences resulting from age and size are more striking, as will be seen by reference to table 7. That the fertilizers did not have a greater influence on the sucrose content may have been due in part to the fact that from the outset the plots were fairly fertile. At the same time it is recognized that in general it is difficult to alter the composition of seeds by these means.

Table 8 (fresh-weight basis) shows that the peas of larger grades had a correspondingly higher starch content than the peas of smaller grades, this being true of peas raised in fertilized and unfertilized plots. In other words, the starch content of the peas varied directly with their size. Another regularity noted was the higher percentage of starch in peas of the later harvest. The regularities observed in the starch content of the peas when the determinations were made on

the fresh-weight basis also hold true when the starch content was determined on the oven-dry weight basis (table 8).

With regard to the influence of the various fertilizers on the starch content of the peas, table 8 shows that the 1930 peas from the untreated plot had on an average 9.22 percent of starch, those from the potash-treated plot had on an average 9.18 percent, while the average starch content of the peas from the plots treated with phosphorus and nitrogen was, respectively, 9.22 and 8.52 percent. By reducing these figures to the starch content of the peas from the untreated plot taken as 100, it will be found that the starch content of the peas from the plots treated with potash, phosphorus, and nitrogen is, respectively, 99.5, 100.0, and 92.4 percent. Thus, the nitrogen treatment has here the only significant influence on the starch formation, the effect being characteristic of delayed maturity. The difference is significant with reference to the potash- and phosphorus-treated plots as well as to the untreated plots.

The facts pointed out in the foregoing discussion will be better comprehended when the carbohydrate metabolism in peas is taken into consideration. Let us assume, as is generally assumed for plants, that the carbohydrate metabolism in peas takes place according to the following equations:

(1) $(CO_2 + H_2O) \longrightarrow CH_2O \longrightarrow C_6H_{12}O_6$
     Carbon   Water   Formaldehyde   Glucose
     dioxide

(2) $C_6H_{12}O_6 + C_6H_{12}O_6 - H_2O = C_{12}H_{22}O_{11}$
     Glucose   Fructose   Water   Sucrose

(3) $m(C_6H_{12}O_6) - n(H_2O) = (C_6H_{10}O_5)X$
     Glucose   Water   Starch
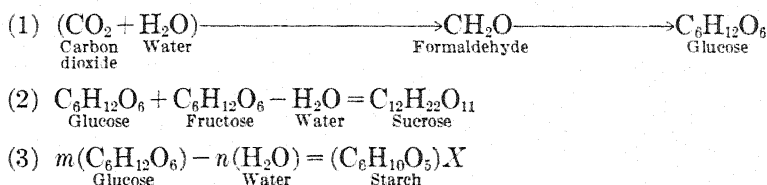
Then the simplest interpretation of the results obtained is that the first sugar to appear, glucose (equation 1), is rapidly changing to sucrose (equation 2). It is for this reason that the proportion of reducing sugars in the peas is quite insignificant. However, it appears that the condensation of reducing substances to sucrose and finally to starch, which is characteristic of maturity, was somewhat delayed in the peas grown on nitrogen-treated soil. This is evident from the fact that the reducing sugars in the 1930 peas from the nitrogen-treated plot (average 0.074) are significantly greater than those in peas from any of the other plots (average 0.043 to 0.056). That the nitrogen has a somewhat retarding influence on the rate of maturity of the peas is also evident from the sucrose content of the peas (table 7). The average sucrose content of the 1930 peas from the nitrogen-treated plot, the check, and the plots treated with potash and phosphorus is, respectively, 3.49, 3.06, 3.14, and 3.31. The phosphorus-treated plot produced peas of a very slightly but significantly higher sucrose content than did the check and potash-treated plots, but inasmuch as peas from the plot given the phosphorus treatment did not exhibit other differences consistently characteristic of either delayed or hastened maturity, this point is in itself unimportant. Thus, only the application of nitrogen had a definite, retarding influence on the rate of maturity of the peas, as evidenced by the sugar content. The sucrose content of the peas harvested on June 2 was smaller than that of the peas harvested on May 28 and is in full harmony with the stated metabolism in the peas.

In the later stages of development glucose is changed chiefly to starch (equation 3). For this reason the more mature peas must necessarily have a larger percentage of starch and a correspondingly smaller percentage of sucrose. That this is actually the case a glance at tables 8 and 9 will show. The fairly high percentage of sucrose and starch in peas grown in the variously treated plots is an indication that these two carbohydrates represent important reserve materials of the peas.

TOTAL NITROGEN

The total nitrogen was determined by Gunning's modification of the Kjeldahl method.

Table 9 shows that when determinations were made on a fresh-weight basis the small-sized peas as a rule had a lower percentage of nitrogen than the large-sized, and that peas harvested early had a lower nitrogen content than those harvested later. On the other hand, when determinations were made on a dry-weight basis the nitrogen content ordinarily decreased with increasing size and age of the peas.

TABLE 9.—*Percentage of total nitrogen in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*

1930 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Nitrogen from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:			*Percent*	*Percent*	*Percent*	*Percent*
1	2	A	0.943	1.013	0.956	0.986
2	2	B	1.004	.967	.935	1.011
		Average	.973	.990	.945	.998
3	3	A	1.003	1.084	1.015	1.033
4	3	B	1.091	1.033	1.055	1.078
		Average	1.047	1.058	1.035	1.055
5	4	A	1.022		1.054	1.041
6	4	B	1.106	1.127	1.110	1.059
		Average	1.064	1.127	1.082	1.050
		Average (1–6)	1.028	1.045	1.031	1.034
Harvest of June 2:						
7	2	A	1.010	1.092	1.102	1.052
8	2	B	1.126	1.029	1.071	1.150
		Average	1.068	1.060	1.086	1.101
9	3	A	1.169	1.235	1.185	1.119
10	3	B	1.246	1.184	1.200	1.286
		Average	1.207	1.209	1.192	1.202
11	4	A	1.215	1.329	1.286	1.296
12	4	B	1.346	1.234	1.296	1.330
		Average	1.280	1.281	1.291	1.313
		Average (7–12)	1.185	1.184	1.190	1.205
		Average (1–12)	1.106	1.121	1.105	1.119

TABLE 9.—*Percentage of total nitrogen in the Alaska pea as affected by different fertilizers; determined on the basis of fresh and of oven-dry weight, 1930 and 1931*—Continued

1931 CROP, FRESH WEIGHT

Sample no.	Grade no.	Plot	Nitrogen from treatment indicated			
			Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:			*Percent*	*Percent*	*Percent*	*Percent*
13	2	A	0.940	0.952	....	0.990
14	2	B	.991	.981	....	.957
		Average	.966	.967	....	.974
15	3	A	1.156	1.126	....	1.156
16	3	B	1.157	1.143	....	1.111
		Average	1.157	1.135	....	1.134
17	4	A	1.299	1.285	....	1.302
18	4	B	1.245	1.274	....	1.272
		Average	1.272	1.280	....	1.287
		Average (13–18)	1.131	1.127	....	1.131

1930 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of May 28:						
1	2	A	4.61	4.81	4.61	4.77
2	2	B	4.77	4.59	4.67	4.81
		Average	4.69	4.70	4.64	4.79
3	3	A	4.41	4.60	4.55	4.75
4	3	B	4.65	4.45	4.52	4.72
		Average	4.53	4.53	4.54	4.74
5	4	A	4.27	....	4.40	4.57
6	4	B	4.59	4.48	4.41	4.58
		Average	4.43	4.48	4.41	4.58
		Average (1–6)	4.55	4.59	4.53	4.70
Harvest of June 2:						
7	2	A	4.25	4.46	4.38	4.61
8	2	B	4.52	4.21	4.17	4.63
		Average	4.39	4.34	4.28	4.62
9	3	A	4.22	4.45	4.25	4.43
10	3	B	4.38	4.22	4.13	4.52
		Average	4.30	4.34	4.19	4.48
11	4	A	4.13	4.50	4.25	4.42
12	4	B	4.32	4.12	4.13	4.47
		Average	4.23	4.31	4.19	4.45
		Average (7–12)	4.30	4.33	4.22	4.51
		Average (1–12)	4.43	4.44	4.37	4.61

1931 CROP, OVEN-DRY WEIGHT

Sample no.	Grade no.	Plot	Check	Potash	Phosphoric acid	Nitrogen
Harvest of June 10:						
13	2	A	4.39	4.38	....	4.43
14	2	B	4.44	4.35	....	4.31
		Average	4.42	4.37	....	4.37
15	3	A	4.34	4.28	....	4.28
16	3	B	4.33	4.17	....	4.21
		Average	4.34	4.23	....	4.25
17	4	A	4.32	4.32	....	4.26
18	4	B	4.24	4.21	....	4.16
		Average	4.28	4.27	....	4.21
		Average (13–18)	4.34	4.29	....	4.28

There were no significant differences in the total nitrogen content of the peas from plots receiving different treatments, which is rather surprising in view of the rather marked increase of sucrose and decrease of starch in peas from the nitrogen plot.

PROTEIN AND NONPROTEIN NITROGEN

The estimation of the protein nitrogen was made according to Stutzer's method (*26*) as applied by one of the writers and reported in previous publications (*12, 13, 14, 15*). The estimation of the nonprotein nitrogen was either made directly by ascertaining the nitrogen in the filtrate from the protein precipitate as obtained by means of Stutzer's copper solution (*11, 16, 26*) or calculated by difference from 100 (tables 10 and 11).

Table 10 shows that the peas of smaller size had a smaller protein nitrogen content than those of larger size.

What is true of the relationship between the different sizes of peas is also true of the relationship between peas of different ages regardless of treatment. For instance, in 1930 grades 2, 3, and 4 of peas from the untreated plot harvested May 28 have 41.21, 48.53, and 51.29 percent of protein nitrogen, respectively (samples 1, 3, and 5), while the corresponding grades of peas harvested June 2 have higher proportions of protein nitrogen. The same holds true of the peas from the potash-, nitrogen-, and phosphorus-treated plots. The nonprotein nitrogen of peas from the various plots stands in reverse ratio to the protein nitrogen, as would be expected. Exactly the same relationships regarding size and age of the peas hold true when the percentages of protein and nonprotein nitrogen are calculated on the dry-weight basis.

The regularities herein reported regarding the decreasing sucrose content and the increasing starch and protein content with greater size and age of the peas are in harmony with the findings of other investigators (*3, 4, 23*). These facts will be better comprehended if the anabolic processes taking place in the pea are taken into account. The nitrates taken up by the pea from the soil are successively converted into nitrites, ammonia, and then into the various organic compounds according to the following scheme:

Nitrates→ nitrites→ ammonia→ mono- and di-amino acids→ acid amides→ polypeptides (peptones, proteoses) (*6, pp. 23–53*)→ proteins.

From this scheme it is plainly evident that in the earlier stages the nonproteins (amino acids, acid amides) are dominant, whereas in the latter stages the proteins, namely, legumin, vicilin, legumelin, and proteose (*18, 19, 20*) are dominant. It would seem that the regularities so conspicuously displayed by the protein nitrogen of peas from the variously treated plots, especially the very considerable and consistent differences in the protein nitrogen between peas of different sizes and of different ages, make this estimation an accurate means of determining the stage of maturity of the Alaska pea.

Although there are no significant differences in the total nitrogen content of the peas from the variously treated plots, some consistent differences occur in the distribution of the nitrogen. The mean percentage of protein nitrogen based on fresh weight of peas from the various plots for 1930 was: Check, 0.614; potash, 0.644; phosphorus, 0.627; and nitrogen, 0.599. The corresponding percentages of nonprotein nitrogen were 0.492, 0.477, 0.485, and 0.520. The differences in amount of these constituents calculated on a fresh-weight basis were too small and too variable to appear important. However, if the percentage of protein or nonprotein nitrogen is calculated on the basis of total nitrogen, some very significant differences between plots are evident. The peas from the potash plot show a protein-nitrogen content that is 56.73 percent of the total, or 1.3 times the nonprotein portion. Calculated similarly, those from the check and nitrogen-treated plots show a protein content of 54.61 and 52.66 percent, or 1.2 and 1.1 times the nonprotein-nitrogen content. The increased proportion of protein nitrogen of the potash-treated peas is not statistically significant, but the decreased proportion of the nitrogen-treated peas is significant when compared with the check or the other treatments. The peas from the phosphorus-treated plot also show a significantly higher proportion of protein nitrogen than do those of the check.

Despite the statistical significance of the differences here discussed, it is doubtful whether the magnitudes are such as to be of great practical importance from the standpoint of culinary quality. The data show, however, a tendency for potash treatment to hasten certain processes that are characteristic of maturity, and for nitrogen treatment to delay those processes.

TABLE 10.—*Percentage of protein and nonprotein nitrogen of the Alaska pea on fresh-weight basis and percentage of protein nitrogen based on total nitrogen, 1930 and 1931*

1930 CROP

Sample no.	Grade no.	Plot	Check			Nitrogen from treatment indicated									
						Potash			Phosphoric acid			Nitrogen		
			Protein nitrogen		Non-protein nitrogen	Protein nitrogen		Non-protein nitrogen	Protein nitrogen		Non-protein nitrogen	Protein nitrogen		Non-protein nitrogen
			Fresh-weight basis	Total nitrogen basis	Fresh-weight basis	Fresh-weight basis	Total nitrogen basis	Fresh-weight basis	Fresh-weight basis	Total nitrogen basis	Fresh-weight basis	Fresh-weight basis	Total nitrogen basis	Fresh-weight basis
			Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Harvest of May 28:														
1	2	A	0.389	41.21	0.554	0.431	40.75	0.600	0.407	42.52	0.549	0.391	39.62	0.595
2	2	B	.431	42.08	.573	.438	45.32	.529	.399	42.61	.536	.437	43.24	.574
		Average	.410	42.09	.563	.435	43.03	.564	.403	42.56	.542	.414	41.43	.584
3	3	A	.487	48.53	.516	.561	51.74	.523	.498	49.01	.517	.452	43.79	.581
4	3	B	.552	50.54	.539	.553	53.48	.480	.532	50.44	.523	.528	48.94	.550
		Average	.519	49.53	.527	.557	52.61	.501	.515	49.72	.520	.490	46.36	.565
5	4	A	.524	51.29	.498				.516	52.05	.496	.516	49.45	.525
6	4	B	.596	53.81	.510	.674	59.82	.453	.619	55.78	.491	.540	53.28	.495
		Average	.560	52.55	.504	.674	59.82	.453	.588	54.36	.493	.540	51.36	.510
		Average (1–6)	.496	48.06	.531	.531	50.22	.517	.502	48.88	.518	.481	46.38	.553
Harvest of June 2:														
7	2	A	.523	51.76	.487	.575	52.69	.517	.591	53.65	.511	.493	46.85	.559
8	2	B	.603	53.54	.523	.572	55.58	.457	.599	55.88	.472	.604	52.48	.546
		Average	.563	52.65	.505	.573	54.13	.487	.505	54.76	.491	.548	49.66	.552
9	3	A	.706	60.43	.463	.785	63.60	.450	.731	61.65	.454	.669	59.82	.450
10	3	B	.802	64.38	.444	.774	65.40	.410	.799	66.59	.501	.806	62.61	.480
		Average	.754	62.40	.453	.779	64.50	.430	.765	64.12	.477	.737	61.21	.465

11	A	4	.806	66.34	.409	.880	66.22	.449	.881	68.24	.405	.851	65.61	.445
12	B	4	.951	70.60	.395	.857	69.42	.377	.917	70.70	.379	.881	66.22	.449
	Average (7–12)		.878	68.47	.402	.808	67.82	.413	.899	69.47	.392	.866	65.91	.447
	Average (1–12)		.732	61.17	.453	.740	62.15	.443	.753	62.78	.453	.717	58.94	.488
	Average (1–12)		.614	54.61	.492	.644	56.73	.477	.627	55.83	.485	.599	52.66	.520

1931 CROP

Harvest of June 10:														
13	A	2	0.446	47.38	0.495	0.448	47.03	0.504				0.474	47.86	0.516
14	B	2	.476	47.97	.516	.489	49.89	.492				.444	46.40	.513
	Average		.461	47.68	.506	.469	48.46	.498				.459	47.13	.515
15	A	3	.719	62.21	.437	.687	60.98	.439				.689	59.58	.467
16	B	3	.724	62.59	.433	.726	63.55	.416				.657	59.14	.454
	Average		.722	62.40	.435	.707	62.27	.428				.673	59.36	.461
17	A	4	.899	69.21	.400	.880	68.52	.404				.892	68.54	.409
18	B	4	.866	69.58	.379	.908	71.26	.366				.859	67.55	.411
	Average		.883	69.40	.390	.894	69.89	.385				.876	68.05	.413
	Average (13–18)		.688	59.82	.443	.690	60.21	.437				.669	58.18	.462

TABLE 11.—*Percentage of protein and nonprotein nitrogen of the Alaska pea on oven-dry weight basis and percentage of nonprotein nitrogen based on total nitrogen*

1930 CROP

Sample no.	Grade no.	Plot	Check — Protein nitrogen (Oven-dry weight basis)	Check — Nonprotein nitrogen (Oven-dry weight basis)	Check — Total-nitrogen basis	Potash — Protein nitrogen (Oven-dry weight basis)	Potash — Nonprotein nitrogen (Oven-dry weight basis)	Potash — Total-nitrogen basis	Phosphoric acid — Protein nitrogen (Oven-dry weight basis)	Phosphoric acid — Nonprotein nitrogen (Oven-dry weight basis)	Phosphoric acid — Total-nitrogen basis	Nitrogen — Protein nitrogen (Oven-dry weight basis)	Nitrogen — Nonprotein nitrogen (Oven-dry weight basis)	Nitrogen — Total-nitrogen basis
			Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Harvest of May 28:														
1	2	A	1.90	2.71	58.79	1.96	2.85	59.25	1.96	2.65	57.18	1.89	2.88	60.38
2	2	B	2.05	2.72	57.02	2.08	2.51	54.08	1.99	2.68	57.39	1.99	2.73	56.76
Average			1.98	2.72	57.91	2.02	2.68	56.97	1.98	2.67	57.41	1.99	2.81	58.57
3	3	A	2.14	2.27	51.47	2.38	2.22	48.26	2.23	2.32	50.99	2.08	2.67	56.21
4	3	B	2.35	2.30	49.56	2.38	2.07	46.52	2.28	2.24	49.56	2.31	2.41	51.06
Average			2.25	2.29	50.47	2.38	2.15	47.39	2.26	2.28	50.28	2.20	2.54	53.61
5	4	A	2.19	2.08	48.71				2.33	2.07	47.05	2.26	2.31	50.55
6	4	B	2.47	2.12	46.19				2.46	1.95	44.22	2.41	2.14	46.72
Average			2.33	2.10	47.45	2.68	1.80	40.18	2.40	2.01	45.64	2.35	2.23	48.61
Average (1–6)			2.18	2.57	51.94	2.30	2.29	40.78	2.21	2.32	51.12	2.18	2.52	53.61
Harvest of June 2:														
7	2	A	2.20	2.05	48.24	2.35	2.11	47.31	2.35	2.03	46.35	2.16	2.45	53.15
8	2	B	2.42	2.10	46.46	2.34	1.87	44.42	2.33	1.84	44.12	2.43	2.20	47.52
Average			2.31	2.08	47.35	2.35	1.99	45.87	2.34	1.94	45.24	2.30	3.33	50.34
9	3	A	2.55	1.67	39.57	2.83	1.62	36.40	2.62	1.63	38.35	2.65	1.78	40.18
10	3	B	2.82	1.56	35.62	2.76	1.46	34.60	2.75	1.38	33.91	2.83	1.69	37.39
Average			2.69	1.62	37.60	2.80	1.54	35.50	2.69	1.51	35.88	2.74	1.74	38.79

Nitrogen from treatment indicated (spanning the Check, Potash, Phosphoric acid, and Nitrogen treatment columns).

11	4	A	2.74	1.39	33.66	2.98	1.52	33.78	2.90	1.35	31.76	2.90	1.52	34.39
12	4	B	3.05	1.27	29.40	2.86	1.26	30.58	2.92	1.21	29.30	2.96	1.51	33.78
		Average	2.90	1.33	31.53	2.92	1.39	32.18	2.91	1.28	30.53	2.93	1.52	34.09
		Average (7–12)	2.63	1.67	38.83	2.69	1.64	37.85	2.65	1.57	37.22	2.66	1.86	41.07
		Average (1–12)	2.41	2.02	45.38	2.51	1.94	43.27	2.43	1.95	44.17	2.42	2.19	47.34
1931 CROP														
Harvest of June 10:														
13	2	A	2.08	2.31	52.62	2.06	2.32	52.97				2.12	2.31	52.14
14	2	B	2.13	2.31	52.03	2.17	2.18	50.11				2.00	2.31	53.60
		Average	2.11	2.31	52.33	2.12	2.25	51.54				2.06	2.31	52.87
15	3	A	2.70	1.64	37.79	2.61	1.67	39.02				2.55	1.73	40.42
16	3	B	2.71	1.62	37.41	2.65	1.52	36.45				2.49	1.72	40.86
		Average	2.71	1.63	37.60	2.63	1.60	37.74				2.52	1.73	40.64
17	4	A	2.99	1.33	30.79	2.96	1.36	31.48				2.92	1.34	31.46
18	4	B	2.95	1.29	30.42	3.00	1.21	28.74				2.81	1.35	32.45
		Average	2.97	1.31	30.61	2.98	1.29	30.11				2.87	1.35	31.96
		Average (13–18)	2.59	1.75	40.18	2.58	1.71	39.80				2.48	1.79	41.82

SUMMARY AND CONCLUSIONS

The application of superphosphate at the rate of 1,000 pounds per acre (160 pounds phosphoric acid) produced a definite increase in yield of the Alaska pea over the untreated plot, amounting to 24 and 18 percent (fresh weight) for the seasons of 1930 and 1931, respectively. On the other hand, the application of 300 pounds of muriate of potash per acre (144 pounds potash) and of 400 pounds nitrate of soda plus 280 pounds of sulphate of ammonia (116 pounds nitrogen) gave differences in yield, as compared with the untreated plot, which were neither sufficiently great nor sufficiently consistent to permit the drawing of definite conclusions.

Records of direct observations in the field, as well as the very similar maturity indices for the peas from the treated and untreated plots, do not show any perceptible consistent differences in the rate of maturity. This is in harmony with earlier field observations and with reports of other investigators as well as with the chemical analyses of the peas from plots treated with potash and phosphorus. However, the peas from the nitrogen-treated plot showed, on an average for the entire season, a somewhat higher percentage of reducing sugars, a significantly higher percentage of sucrose, and a distinctly lower starch content as compared with the peas from the untreated plot.

Although the various treatments were without consistent effect on content of total nitrogen, the peas of the nitrogen-treated plot exhibited a slightly but significantly smaller proportion of protein nitrogen than peas from the check or any of the other plots. On the other hand, peas grown on the potash-treated and phosphorus-treated plots showed a larger proportion of protein than did the check, suggesting more advanced or hastened maturity as a result of the treatments. However, the phosphorus treatment was followed by no noticeable difference in percentage of any of the carbohydrates, of ash, or of ether extract. Thus, it seems safe to conclude that the maturity and hence the quality of the peas were unaffected by the superphosphate. The peas from the potash plots, on the other hand, exhibited slightly and significantly higher ash and ether-extract content than did the checks and those receiving the other treatments. Potash treatment was accompanied by increases in 3 of 5 constituents which are known to increase with maturity. Only the two carbohydrate fractions, sucrose and starch, which are believed to be of primary importance in determining quality and maturity, were unaffected. Thus it seems that potash does have a slight tendency to hasten the maturity of the pea. This tendency, however, is so very slight, the differences are of such very small magnitude, that they are of questionable practical importance.

Stages of maturity are characterized by distinct and marked differences. In general, more mature peas, i.e., larger peas or peas of the same size but of later harvest, have a larger percentage of ash, of ether extract (fat), total nitrogen, starch, and protein nitrogen, and a lower percentage of sucrose. On a dry-weight basis the percentages of all constituents except starch and protein nitrogen decrease as maturity progresses.

Muriate of potash and superphosphate applied singly are apparently without consistent and significant effect upon the rate of development or the quality of Alaska peas as indicated by partial chemical analysis

or behavior in the field.   Readily available nitrogen tends to delay maturity appreciably, as is indicated by the higher sugar and lower protein and hydrolyzable-polysaccharide content.

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ABNORMALITIES IN THE FLOWER AND FRUIT OF CAPSICUM FRUTESCENS [1]

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INTRODUCTION

Relatively few cases of abnormalities in *Capsicum frutescens* have received attention in the literature. The cases reported are scattered over a period of more than half a century. No detailed historical study has apparently been made to differentiate between abnormalities infrequently occurring in the flower and the normal flower itself, and only in the paper by Harris (6)[3] has any such work been reported on those more frequently found in the fruit. It is the object of this paper, therefore, to review the literature confined to abnormalities in the genus *Capsicum*, and to present a more detailed study of those occurring in both the flower and the fruit.

The work reported here was done in connection with some studies that are being carried out on fruit setting in the pepper (*Capsicum frutescens* L.) as influenced by certain environmental factors.

LITERATURE REVIEW

The earliest and yet perhaps the most extensive investigation concerning abnormalities in the genus *Capsicum* is that of Terracciano (15), who noted the changing of stamens into carpels and a growing together of these with the pistil in the flower of *C. grossum*. He also reported two types of abnormalities in the fruit of *C. annuum*. In one form the walls of the fruit, terminated by a hollow tubiform style open at the top, showed five protuberant evaginations. Internally the fruit produced no seeds but contained five similar fruits, likewise without seeds and with short styles having almost perfect stigmas, disposed upon the apex of the thalamus.

Mottareale (10) reported adesmy of the carpels in several cases, and stamens that were both larger and smaller than the corolla pieces. He also saw diaphysis and ecblastesis of the fruit. Both abnormalities were in a few cases seen in the same fruit. Mottareale cites Terracciano as having established that the staminate vasal collar develops externally like the corolla and is capable in *Capsicum grossum* of generating carpels. Not only are the carpels formed at the alternating petalous points, transformation of the normal stamens, but also at the epipetalous points, forming small fruits by development and also by a change of the basal teeth with which every stamen is provided.

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Heckel (8) observed pistillody of the stamens and a coalescence of them with the pistil in flowers of *Capsicum annuum*. He also reported that abnormalities in the fruit were relatively common. He found only a single carpel in some, while in others there were present more or less well formed fruits.

Some unusual types of abnormalities have been reported, chief among which is one by Schilberszky (13). From a common pedicel two individual fruits of *Capsicum annuum* arose and were grown together at the lower side in an angle of about 80°. Both fruits were equal in size and normally developed. The calyx which usually has 5 points, in this case had 10 and was forced between the single fruits in such a manner that it formed an obtuse angle.

Gallardo (2) has described a case in *Capsicum annuum* where internal tuberlike formations as large as 2.5 cm in diameter appeared, which he thought resulted from seeds becoming swollen. Welter (17) found that very often the seeds of *C. annuum* sprouted in the fruit.

Viviand-Morel (16) reported a small internal fruit in the pepper which originated from the apex of the axis, while Halstead (4, 5) noted an almost identical case of a miniature included fruit that arose from the apex of the fleshy columella on which the seeds are borne. It resembled a normal fruit very much and was green. Raymondaud (12) also noted a small, globular, olive-shaped internal fruit in *Capsicum annuum*.

Penzig (11, v. 2, p. 174) reported, after a long study, that abnormalities occurred rather frequently in the fruit of the genus *Capsicum*. Penzig (11, v. 3, p. 77) cites Borbás (1) as having found central prolification of the fruit in the same genus. This is also borne out by the findings of Harris (6). Irish (9) in his extensive work with the pepper has reported similar abnormalities in the fruit.

Sturtevant (14) reported that sweet peppers were subject to the development of a berry or berries within the berry. These enclosed fruitlike bodies were in some cases found to produce a few seeds.

MATERIALS AND METHODS

During 1932–33 two crops of peppers were grown in the experimental greenhouses of the Department of Vegetable Crops at Cornell University. Plants from which material was collected were of the World Beater variety grown both in bank sand, to give a low-nitrogen series, and in soil high in organic matter to which sodium nitrate was added at weekly intervals to give a high-nitrogen series, in 1-gallon glazed crocks. One plant was placed in each crock and grown under two controlled temperature conditions, medium 60°–70° F., and warm 70°–80°. Fresh material was observed under a wide-field binocular microscope. Slides were made of paraffin sections, the material being killed and fixed in a solution made as follows: Solution A, 1 g chromic acid and 10 cc glacial acetic acid in 90 cc of water; solution B, 40 cc of commercial 40 percent formalin added to 10 cc of water (Karpechenko's chromo-acetic). Equal parts of the two solutions were mixed at the time they were used. Heidenhain's iron-alum haematoxylin was found very satisfactory for staining all tissues studied in the histological part of the investigation.

TERATOLOGICAL TRANSITIONS IN THE FLOWER

The normal flower of *Capsicum frutescens* (fig. 1, *D*) occurs either singly or 2 or 3 together in the axils of branches. It has a minutely 5-lobed calyx and a rotate corolla that is also 5-merous. The syle is linear, straight, longer than the stamens, and terminated by a subcapitate stigma. There are 5 stamens attached to the ovary
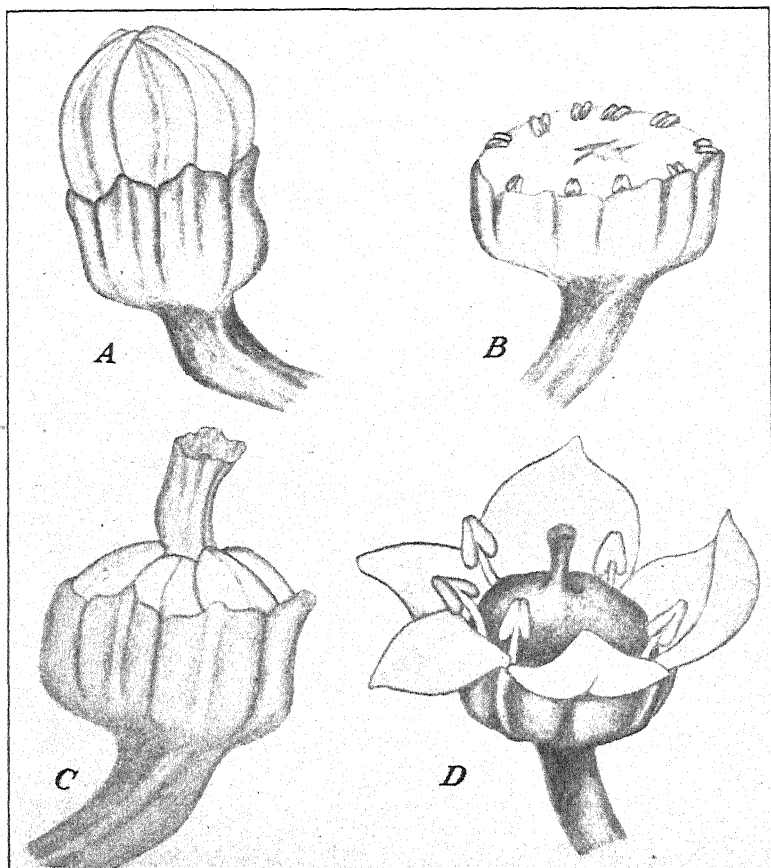
FIGURE 1.—*A*, Normal bud 1 day prior to anthesis; *B*, abnormal bud devoid of pistil (note contorted area where pistil usually is attached) and stamens devoid of filaments; *C*, young abnormal bud with protruding style, to be compared with *A*; *D*, normal flower on date of anthesis.

near the base of the corolla having heart-shaped anthers that dehisce by longitudinal splitting. The ovary is usually 3-celled.

In the case of the abnormal flower (fig. 2, *A*) which is ordinarily easily recognized, some interesting teratological changes sometimes take place. The calyx may undergo partial metamorphosis, thus exhibiting almost unconceivable transitions. One very abnormal case was noted in this study when a calyx lobe enlarged and assumed the appearance of an almost fully developed petal (petalody), while another calyx lobe on the same flower developed into a short pistil (pistillody). According to Gray (*3, p. 174*) pistillody from parts

other than the stamens rarely occurs. Some abnormal flowers have normal calyxes, i.e., 5-lobed, others have as many as 8, while still others, as was noted by Mottareale (*10*) and the writer, may be completely deprived of the calyx.

A great variation was found in the stage of development of the flower at which the transformations occur as well as the time taken
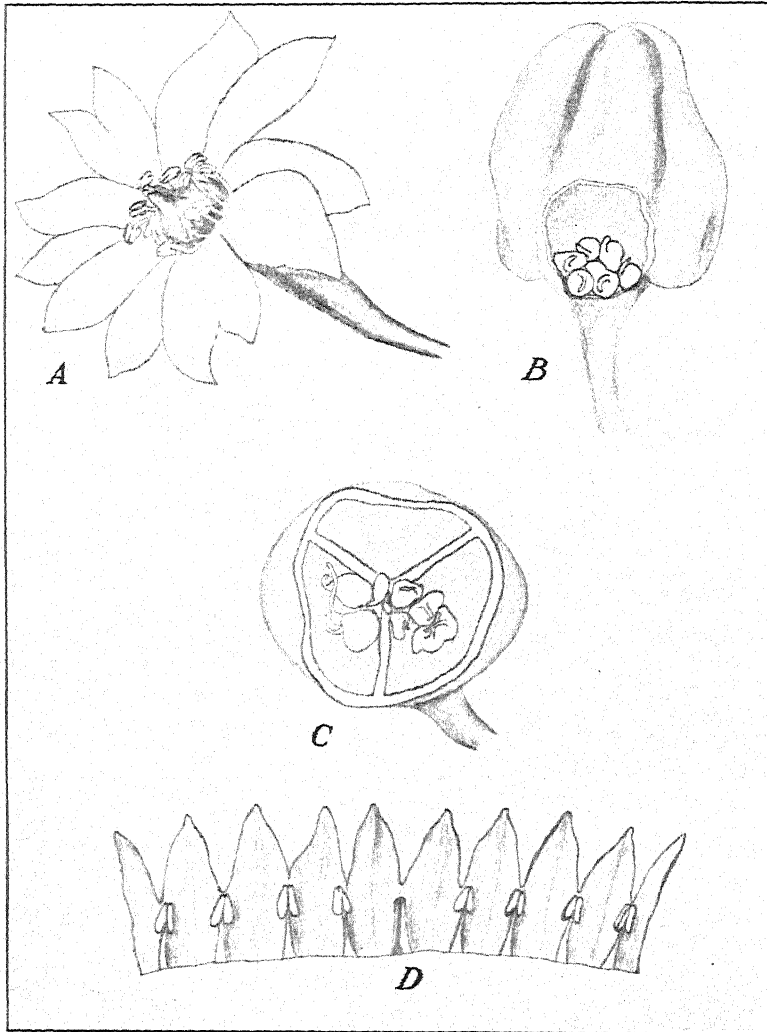
FIGURE 2.—*A*, Abnormal flower with many petals, some divided, and 13 stamens; *B-C*, mature fruits showing internal fruitlike bodies on receptacle; *D*, abnormal corolla with 9 stamens attached, of which 8 are normal, and 1 lacks the anther, but has instead a round hole extending to bottom of filament.

for their completion. Staminody may be initiated in the young bud stage and completed during that time, or it may not start until about the time of anthesis. The abnormal phenomenon may cease at this stage, leaving a contorted-appearing organ or after a time complete itself.

As is shown in figure 3 the abnormal corolla does not shed within 2 or 3 days or become withered, like that of the normal flower. It persists until the ovary develops to the point that the basal collar is split, thus allowing the corolla to drop.

While the majority of abnormal flowers contain from 7 to 9 stamens, occasionally there may be more. In figure 2, *A* is shown a flower with 13 normal stamens, whereas in figure 1, *B*, is shown another flower with 10 anthers completely deprived of their filaments. In figure 2, *D*, is a flower with 9 stamens, 8 normal ones and another devoid of its anther but with an opening at the apex that extends to the bottom of the filament. A similar case of the latter abnormality was reported by Mottareale (*10*).

Stamens sometimes undergo partial retrogressive metamorphosis thus causing some of them to unite with the petals, and they may even
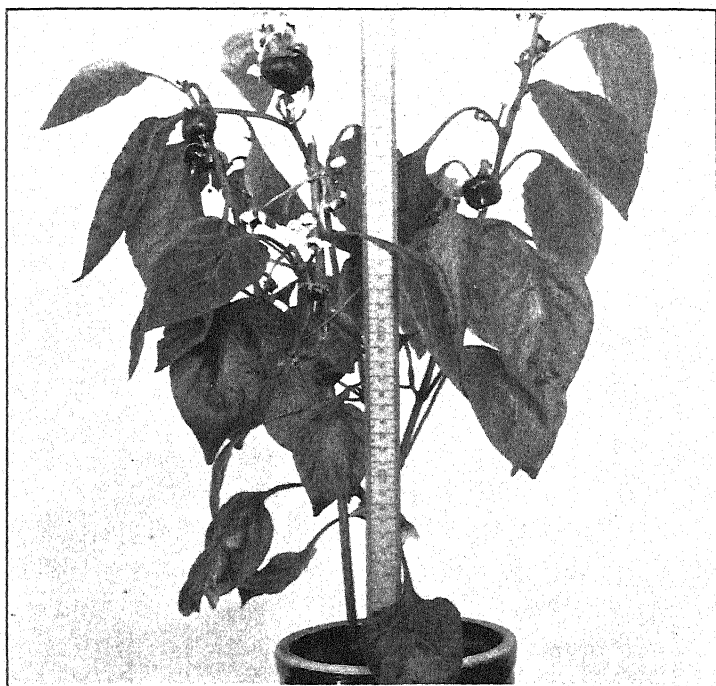
FIGURE 3.—Plant of *Capsicum frutescens* showing abnormal corollas which persist in being attached.

take the appearance of petals but may not complete the transition to petalody. However, this completion was observed during the course of this study.

The abnormal style is perhaps the most easily recognized flower part. Normally it can be detected in the early bud stage by its unusual protrusion above the tightly closed petals. It is generally short and broad, and often severely contorted as is shown in figure 1, *C*. Complete anthesis of such flowers is delayed, and owing to the early emergence of the stigmatic surface beyond the stamens, the chances for self-pollination are very small. Owing to this and to the fact that sometimes such stigmatic surfaces fail to become receptive, the ovules are not fertilized and the fruits develop

However, cross-pollination and perhaps self-pollination, evidently take place in a few cases. A point of great interest at this stage is the behavior of such styles with reference to their development and abscission. Normal styles at the time of anthesis are rarely more than 1 mm in diameter near the base and 5 mm in length. The styles usually dry up and drop soon after fertilization has taken place. Very often the style of an abnormal flower has become 2 to 2.5 mm in diameter and 7 to 9 mm in length by the time the flower opens. It does not absciss but invariably remains attached to the ovary, develops chlorophyll, and increases in size with the ovary until maturity, at which time it takes on the normal red color. The development of the various stages of the abnormal style is shown in figure 4. Occasionally flowers develop devoid of styles as can be seen in figure 1, *B*. There is usually a more or less contorted cavity at the apex of the ovary in such fruits. The ovary may in some cases split longitudinally into a rosette of stylar portions, some separate, other united. Each portion may develop a stigmatic surface which
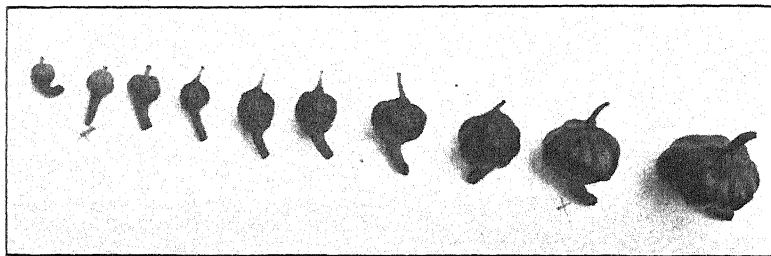
FIGURE 4.—Development of the abnormal style; it does not absciss but continues in growth with the ovary. Corollas have been removed from the larger fruits.

appears to be very abnormal. Such conditions are not found very frequently, however, and the stage to which they develop depends on the age of the ovary.

TERATOLOGICAL TRANSFORMATIONS IN THE FRUIT

Harris (7) in working with okra, states:

Among the various phenomena that may be included by the teratologists under the term "prolification of the fruit", one of the most interesting is the production of a more or less completely formed second fruit inside the first. Generally, the included "fruit" is distinctly abnormal in character, often reduced to a whorl, or a series of whorls, of irregularly formed and usually sterile carpels.

The fruitlike structures in *Capsicum frutescens* apparently have no definite form but vary from an irregular contorted body through an almost perfectly formed sterile fruit, comparable in shape with the one in which it is produced, to linear bodies from a few millimeters to 2 cm in length, some of which are terminated by a minute style. A few of the fruitlike bodies noted, however, contained no styles, as is shown in table 1. It is also apparent from these data that other bodies contained more than one style. Several bodies had as many as three styles, and they were not always normal in form as some were very much fasciated. In one case there were four styles. One style, however, was practically always found attached at the apex of the body while the others were usually formed near the apex or on the side. This is shown in figure 2, *B*, *C*.

TABLE 1.—*Distribution of abnormalities in the fruits of Capsicum frutescens*

Lot no.	Date fruits were harvested	Temperature under which plants were grown	Fruits harvested	Normal fruits	Abnormal fruits	Fruits abnormal	Internal abnormalities	Abnormalities having styles	Styles contained	Seeds in abnormal fruits
		° F.	Number	Number	Number	Percent	Number	Number	Number	Number
1	Dec. 7, 1932	60–70	191	188	3	1.57	71	50	76	306
2	Dec. 26, 1932	70–80	129	123	6	4.65	55	42	78	14
3	Jan. 11, 1933	70–80	213	204	9	4.22	69	43	54	34
4	Jan. 21, 1933	60–70	175	170	5	2.85	33	25	61	12
5	Feb. 4, 1933	70–80	163	156	7	4.29	37	28	54	32
6	Feb. 16, 1933	60–70	228	217	11	4.82	34	16	32	28
7	Feb. 28, 1933	60–70	195	187	8	4.10	22	15	21	95
8	Mar. 19, 1933	70–80	142	132	10	7.04	36	19	32	1
9	May 6, 1933	70–80	223	216	7	3.13	15	10	21	286
10	May 19, 1933	(a)	216	202	14	6.48	50	40	86	9
11	July 13, 1933	(a)	147	125	22	14.96	55	41	81	75
Total			2,022	1,920	102	5.04	477	329	596	886

a Temperature uncontrolled.

The data in table 1 show a seasonal distribution of the abnormalities in the fruits. Even though the percentage of abnormal fruits was not unusually high at any one time, abnormal fruits were present throughout the duration of the experiment. The number of internal bodies was in no way constant. In fact, the determination of such was sometimes rather difficult because of the varying degrees of fusion of the members of the outer whorls and the small size of those of the inner ones. Harris (*6*) counted the number of styles and used this as a criterion of the number of internal abnormalities in *Passiflora*, but the writer found the method unsatisfactory in the present study because some of the included bodies contained more than one style. In the majority of cases reported here, counts were made under a wide-field binocular microscope using a dissecting needle to separate the sometimes much entangled fruitlike structures. Some fruits contained only 1 of these structures, while others had as many as 18 to 20, and in one case 22 of such abnormalities were noted. They completely filled the fruit cavity.

HISTOLOGICAL DIFFERENTIATIONS BETWEEN PARTS OF THE NORMAL FLOWER AND FRUIT AND EXISTING ABNORMALITIES

Since no previous work has been reported that differentiates histologically between abnormalities infrequently occurring in the flower and the normal flower itself, attention has naturally been focused on this relationship here. Figure 5, *A* and *B*, shows cross sections of a normal style and the style of an internal fruitlike body. Both are similar in some tissues, yet as a whole their development and tissue arrangement resemble each other but very little. The style of the abnormal fruit failed to develop a stylar canal. This fact may offer a partial explanation for the failure of the internal abnormal fruitlike bodies to become fertilized, and to form seed even when pollen has been applied to the stigmalike surface. Some abnormal styles were found to contain only 1 vascular bundle while others were frequently noted that contained from 6 to 8. The usual number of vascular bundles found in the normal style is 4.

The conducting tissue through which the pollen tubes pass is usually found immediately surrounding the stylar canal as is shown in figure 5, *A*. In the abnormal style it may be completely absent or found to
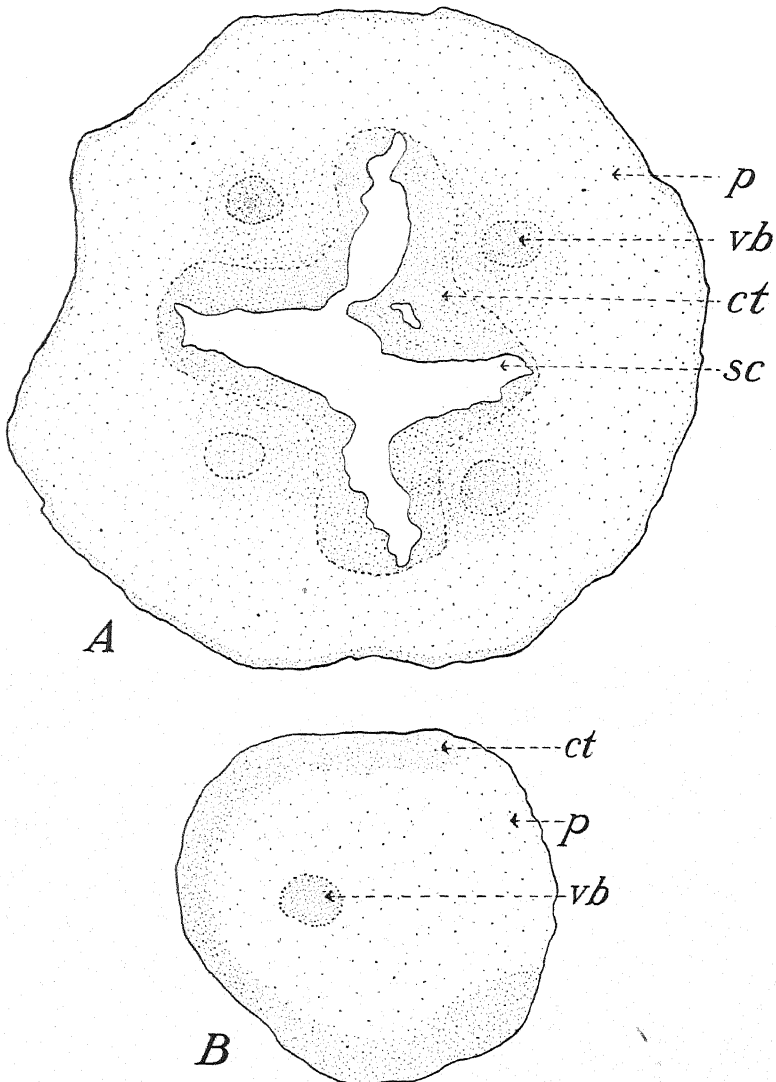
FIGURE 5.—*A*, Cross section of normal style: *p*, Parenchyma; *vb*, vascular bundle; *ct*, conducting tissue; *sc*, stylar canal. *B*, Cross section of abnormal style of internal fruitlike body. ×178.

one side and sometimes mixed in with the parenchyma tissue. This can be seen in figure 5, *B*.

Harris, (*6, p. 137*) in his mention of the histological similarity existing between carpellike bodies and the wall of the fruit, writes:

Both show the exceedingly heavy epidermis and the large-celled parenchymatous ground tissue. The bodies show one or more vascular bundles similar to

those of the wall of the fruit.   When fresh sections are examined, numerous chloroplasts are seen in both but they are generally more abundant in the wall of the fruit.

That the wall of the internal fruitlike body is similar histologically to the wall of a normal fruit is shown in figure 6, *A* and *B*.   There are
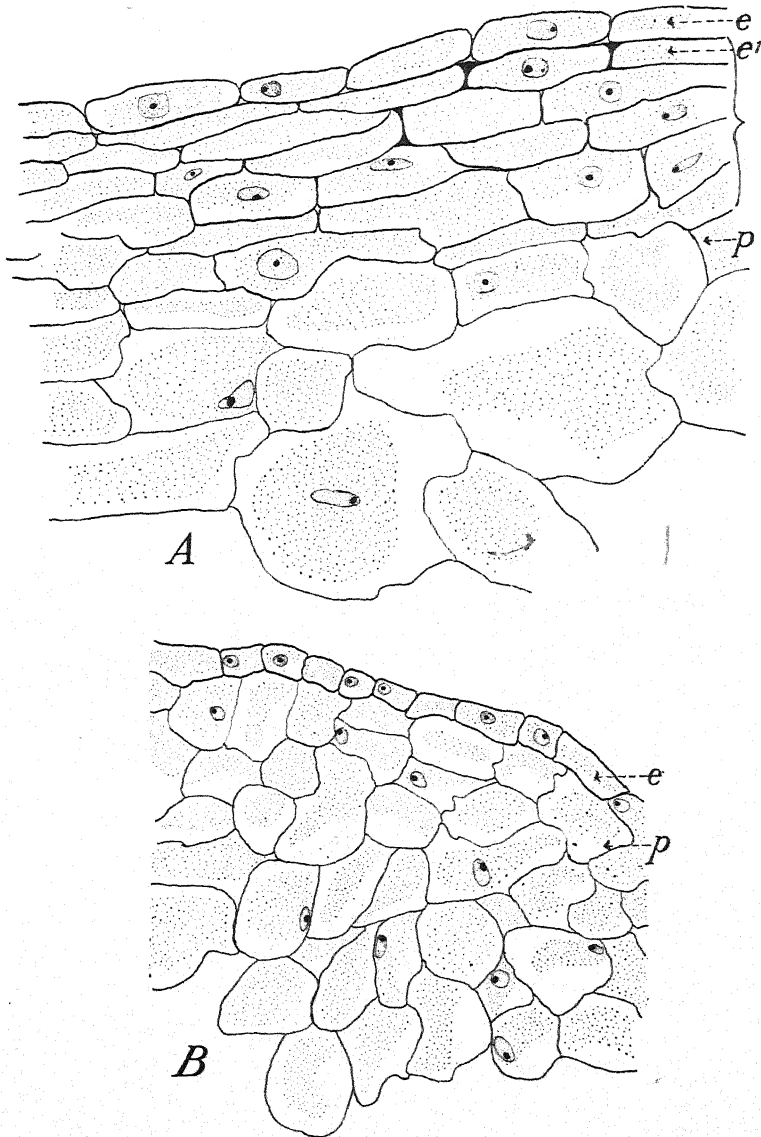
FIGURE 6.—*A*, Cross section of wall of normal fruit: *e*, Epidermis; *e′*, epicarp; *p*, parenchyma.   *B*, Cross section of wall of internal fruitlike body.   × 750.

some differences noted, however, in that the wall of the fruit contains several layers of cells constituting the epicarp which are absent in the wall of the fruitlike body.   The former also contains much larger

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parenchyma cells than does the latter. The chloroplasts and vascular bundles were similar in both cases.

DISCUSSION

The question as to what factors cause the development of abnormalities has probably arisen in the minds of all workers who have reported them, but up to the present time explanations are lacking. Mottareale (*10*) is the only previous worker who has attempted to correlate environmental conditions with the existence of these abnormalities. He attributed the abnormalities found in the flower and fruit of *Capsicum annum* and *C. grossum* to short periods of cold weather under which the plants were grown. This explanation, however, does not seem altogether feasible, since he later repeated the same treatment twice and was unable to obtain similar results. Work of the writer tends to disprove the idea that low temperature is the only factor that results in the initiation and production of abnormalities. Those that are reported here were produced on plants grown under controlled medium (60° to 70° F.) and high (70° to 80°) temperatures in greenhouses during the winter of 1932, and under uncontrolled temperatures (70° to 110°) in greenhouses during the summer of 1933. This does not prove, however, that abnormalities would not be initiated under still lower temperatures than those used in this study. The data in table 1 show that the number as well as the percentage of abnormal fruits was greater during the summer under high uncontrolled temperatures than at any other time of the year. This fact suggests that high temperature is more effective in producing abnormal fruits than is medium temperature. In addition, the results of examination of a large number of flowers during the same periods agree with these findings in regard to the fruits.

From the beginning of this study indications seemed to show that a competition for nutrients or elaborated food between the rapidly growing normal plant parts may be the cause of the initiation of abnormalities since they appeared first on plants in the low-nitrogen series. Soon afterwards, however, several abnormal fruits and one abnormal flower were noted on plants in the high-nitrogen series. In fact, by the time the experiment terminated plants in the high-nitrogen series had produced more abnormalities than those in the low-nitrogen series, probably because the former produced more flowers and fruits.

SUMMARY

A review of the literature concerning abnormalities in the genus *Capsicum* and a detailed study of those occurring in both the flower and the fruit are reported here.

A very rare case was noted in which two calyx lobes on the same flower assumed different teratological transformations, namely, pistillody and petalody.

There is no definite stage in the formation of the flower at which the transitions are initiated or completed.

The abnormal corolla does not drop or wither within 2 or 3 days but persists until the ovary develops to the point that the basal collar splits and this allows the corolla to drop.

Stamens may undergo partial retrogressive metamorphosis, thus causing some of them to unite with the petals, and may even take

the appearance of such, but may not complete the transition to petalody.

The abnormal style can commonly be detected in the early bud stage by its unusual protrusion above the tightly closed petals. It does not absciss but remains attached to the ovary, develops chlorophyll, and increases in size with the ovary until maturity, at which time it takes on the normal red color.

The internal fruitlike bodies are usually distinctly abnormal in character. Some have no style, while other have as many as 3, and in one case 4 were noted.

The number of internal abnormalities was in no way constant, as they ranged all the way from 1 to 22.

Histological differentiations are made between parts of the normal flower and fruit and existing abnormalities.

Both the style of a normal flower and that of an interal fruitlike body are similar in some tissues, yet as a whole their development and tissue arrangement parallel each other but very little. The fruitlike bodies failed to develop any indications of a stylar canal.

Histological similarities between the wall of the internal fruitlike body and the wall of the fruit are interesting. Both have a heavy epidermis, large parenchyma cells, chloroplasts, and vascular bundles. There is one very distinct difference, however, in that the fruit wall contains several layers of cells, which make up the epicarp and which are absent in the wall of the carpel like body.

While both abnormal flowers and fruits were produced more abundantly on plants growing under high and high uncontrolled temperatures, they appeared to some extent on plants under medium temperature.

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SOIL TREATMENT IN RELATION TO CLUBROOT OF CABBAGE [1]

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INTRODUCTION

The investigation herein described is a continuation of work carried on by Wellman [2] during the years 1924 to 1928 inclusive. In a report of his studies he noted that the hydrate was the most effective compound of calcium used to control clubroot of cabbage. In 1928 he secured good control of the disease on severely infested soil in Kenosha County, Wis., with a 2-ton treatment of this chemical. In 1930 and 1931 field treatments were carried out by the writers on infested soil in the same locality. Since in these cases even heavier treatments of the soil with calcium hydrate had little effect upon clubroot infection, further studies were undertaken to determine, if possible, what factors might influence the effectiveness of liming materials.

Previous work on the relation of soil reaction to infection by the clubroot organism (*Plasmodiophora brassicae* Wor.) has been reviewed by Wellman. Chupp [3] and Wellman [4] both succeeded in completely inhibiting infection in the greenhouse by applying calcium hydrate in sufficient quantities to make the soil reaction slightly alkaline. In the field experiments results were not always consistent. Ravn [5] secured considerable reduction in infection by the use of finely divided $CaCO_3$ and still better control with a mixture of $CaCO_3$ and CaO. It is important to note, however, that in some cases severe infection occurred and he was unable to explain the variation. Chupp [6] failed to secure effective control in the field with calcium hydrate in the early part of the season, although late-season plantings on the same soil were much less severely infected. Wellman secured complete inhibition of infection in the greenhouse with a 2-ton application of calcium hydrate, but this treatment did not preclude root infection in the field, although it did give very good control. In an examination of the numerous reports of liming experiments with clubroot, one is impressed most by the lack of consistent success in disease control in the field.

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FIELD EXPERIMENTS

The field experiments discussed herein were conducted on two badly infested fields. One of these was located near Somers, Kenosha County, Wis., and will be referred to hereafter as the Miller plot. The other was located near Franksville, Wis., and is designated as the Franksville plot. The Miller plot was nearly level except for a low swale which passed through it. The soil was a well-drained Carrington silty clay loam, quite uniform in consistency. The Franksville plot had a slight irregular slope, and the soil was a Clyde silty clay loam. The commercial cabbage crop of 1930 on this area was quite generally infected, but the infection was noticeably least severe on the lower portion of the slope.

EXPERIMENTS ON THE MILLER PLOT

1930 TRIALS

A heavy application of manure was made before plowing. After the land was prepared, 3–10–10 fertilizer was disked in thoroughly at the rate of 1,000 pounds per acre. The liming materials were applied and disked into the soil on June 9 and cabbage (*Brassica oleracea capitata* L.) plants were set on June 20. Two types of hydrate were used. One, which will be referred to as calcium hydrate, had been prepared from limerock consisting of about 95 percent calcium carbonate. The other, dolomitic hydrate, was prepared from dolomitic limestone which contained approximately equal amounts of calcium carbonate and magnesium carbonate.

At the time of liming, the soil was relatively dry and it remained so until transplanting. For 2 weeks after transplanting there was no rain, and the only addition of water during this period was that applied around the plants when they were set. Thus the conditions following transplanting were, according to Monteith,[7] those least favorable for infection, although the "watering-in" of the plants would, according to Wellman[8], be sufficient to favor a certain amount of infection.

The reaction of the soil before lime was applied was pH 5.5, and at the end of the season in the untreated area it was pH 5.8. In the 2-ton calcium hydrate treatment the reaction of the soil changed from pH 5.5 to 7.1, and in the 4-ton treatment to 7.4. In the case of dolomitic hydrate the pH of the soil was somewhat higher, shifting from 5.5 to 7.2 in the 2-ton treatment and to 7.6 in the 4-ton treatment.

General infection became evident by the flagging of the plants during the middle of the day about 8 weeks after they were transplanted. This distinguishing symptom of clubroot infection was apparent over most of the plot. At this time there appeared to be little difference between the treated and untreated areas. It is worthy of note, however, that the cabbage on the area treated with dolomitic hydrate, 4 tons per acre, produced somewhat larger plants than did those on the areas receiving the other treatments.

After the yield data were taken, a large number of plants in each of the treated and untreated areas were pulled and the roots examined to

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[7] MONTEITH, J., JR. RELATION OF SOIL TEMPERATURE AND SOIL MOISTURE TO INFECTION BY PLASMO DIOPHORA BRASSICAE. Jour. Agr. Research 28: 549–562, illus. 1924.
[8] WELLMAN, F. L. See footnote 2.

determine the amount and severity of the disease. All the roots examined were more or less clubbed. There was no measurable difference in the extent of infection in the treated and the untreated areas except that in the former the clubs were usually intact, whereas in the latter they were usually completely decayed, indicating that infection has taken place earlier.

Less than 2 percent of the plants in the treated areas produced marketable heads. The amount of practical control was negligible, and no measurable differences in yield between the four treated areas and the untreated area were secured except on the low area in one portion of the field. In this area, which was favored with a higher level of soil moisture during the growing season, some evidence of the action of the lime as a disease inhibitor was observed. The plants made much better growth as the season progressed, and when the root systems were examined at the end of the growing period a large percentage of plants from the limed portion showed complete inhibition of infection, while in the unlimed portion infection was general.

It is evident from the results of this season that even though calcium and dolomitic hydrate were applied in amounts sufficient to change the reaction of the soil to well above pH 7.0, the amount of infection in most of the plot was not materially affected. This suggested that seasonal or soil conditions influenced the effectiveness of the hydrate in some way not indicated by the pH reading.

1931 TRIALS

In 1931 portions of the same treated and untreated areas of the Miller plot were replanted without further application of liming materials. Another portion of the same field was laid out for further trials. To this various amounts of calcium hydrate and Agstone (finely ground dolomitic limestone) were applied in the autumn of 1930. The field was planted to cabbage on June 1, 1931. Rainfall was again below normal during the growing season.

TABLE 1.—*Effect of various soil treatments upon soil reaction and yield of cabbage on a clubroot-infested soil; Miller plot, 1931*

Material applied	Rate of application per acre	Date of application, 1930	Soil reaction			Yield, September 1931		
			August 1930	June 1931	September 1931	Plants	Marketable heads	Weight of heads per acre
	Tons		*pH*	*pH*	*pH*	*Number*	*Percent*	*Tons*
None	0		5.5	5.8	5.8	2,110	1	0.34
Dolomitic hydrate	2	June 9	7.2	7.1	7.0	246	6	.25
	4	...do	7.6	7.4	7.4	243	11	.34
	2	...do	7.0	7.0	6.8	249	4	.12
	4	...do	7.4	7.2	7.2	240	5	.15
Calcium hydrate	1	Oct. 22	5.5	7.2	7.0	961	17	1.42
	2	...do	5.5	7.3	7.1	968	23	2.08
	1	...do	5.5	7.1	7.1	971	28	1.98
	2	...do	5.5	7.4	7.2	965	26	2.98
Agstone	4	...do	5.5	7.4	7.2	462	3	.17
	8	...do	5.5	7.8	7.6	471	4	.24

In table 1 is shown the reaction of the soil in the various plots in August 1930, at the time of transplanting in June 1931 and at the end of the season in September 1931. At the time the plants were set the

soil in all treated plots was neutral or above. In fact there was then little difference in soil reaction between the plots that had been treated with Agstone and those that had been treated with hydrate in October 1930, or between the plots treated with hydrate in June 1930 and those treated with hydrate in October 1930. In spite of this there was no appreciable reduction in clubroot infection in the hydrate-treated plots of June 1930, nor in the Agstone-treated plots of October 1930. While infection was general in the hydrate plots of October 1930, there were distinct benefits from these treatments, as is shown by the percentage of marketable heads produced. Even in these cases, however, a commercially profitable degree of control was not attained.

EXPERIMENTS ON THE FRANKSVILLE PLOT

1931 TRIALS

The Franksville plot was plowed in the autumn of 1930, and to certain portions of it 1½ and 2½ tons of calcium hydrate per acre and 4 and 8 tons of Agstone per acre were applied on October 22, 1930. Further treatments of calcium hydrate were applied to other portions at the rate of 1½ and 2½ tons per acre the following spring, one series of treatments being made on April 25, as early as seasonal conditions permitted, and the other on June 2, shortly before the entire plot was planted on June 5.

TABLE 2.—*Effect of various soil treatments upon soil reaction and yield of cabbage on a clubroot-infested soil; Franksville plot, 1931*

Material applied	Rate of application per acre	Date of application	Soil reaction		Yield, September 1931	
			September 1930	September 1931	Plants	Marketable heads
	Tons		*pH*	*pH*	*Number*	*Percent*
None	0		6.2	6.3	2,078	2
Agstone	4	Oct. 22, 1930	6.2	7.0	864	3
	8	----do-------	6.2	7.4	870	5
	1½	----do-------	6.2	7.0	2,074	25
	2½	----do-------	6.2	7.2	2,170	34
Calcium hydrate	1½	Apr. 25, 1931	6.2	7.0	571	27
	2½	----do-------	6.2	7.2	507	37
	1½	June 2, 1931	6.2	7.1	574	30
	2½	----do-------	6.2	7.2	572	41

In table 2 the effect of the various treatments upon soil reaction and upon yield of marketable heads is given. All treatments changed the reaction to neutral or above. The heavier Agstone treatment gave the highest pH reading, but there was little effect upon clubroot as measured by the percentage of plants which produced marketable heads. The calcium hydrate treatments as a whole were much more effective, the heavier of these treatments raising the pH slightly and giving somewhat higher percentages of marketable heads. None of the hydrate treatments gave what might be considered a commercially successful control. Although there was some reduction in the amount of clubbing as compared with that on the untreated area and on the areas treated with Agstone, the roots of most plants were more or less infected.

The results obtained in 1931 on the Miller plot suggested that fall application might be more effective since in this case it was more influential than the treatment of the spring of 1930. Chupp [9] attributed increasing effectiveness of hydrate during the current season to the time required for the hydrate to become active. In the Franksville experiment a direct comparison between fall, early spring, and late spring applications of hydrate is made. No indication is given that a long interval between application and planting enhanced the inhibition of infection. In fact, the last application, made 3 days before planting, gave slightly better results, although the actual differences are negligible.

1932 TRIALS

The Franksville plot was replanted in 1932, with no further additions of lime. The purpose was to determine whether or not the treatments of 1931 would affect clubroot infection the second season after application. The rainfall was somewhat heavier and more evenly distributed than in 1931. As the season progressed it became increasingly evident that the disease was less severe in the lower portions of the field regardless of treatment. In the final collection of data the percentage of infection and marketable heads was estimated in high and low portions of the same treated area where such an area traversed both levels. The data thus secured are given in table 3. In general the amount of infection was slight in the low portions of the plot and severe in the high portions, irrespective of treatment, and there was no direct correlation between soil reaction and inhibition of clubroot.

TABLE 3.—*Effect of various soil treatments upon soil reaction, yield, and clubroot infection, Franksville plot, 1932*

Material applied	Rate of application per acre	Date of application	Elevation	Soil reaction, September 1932	Marketable heads	Clubroot infection Severe	Clubroot infection Slight
	Tons			*pH*	*Per-cent*	*Per-cent*	*Per-cent*
None	0		High	6.9	0	100	0
	0		do	6.9	1	98	0
	0		Low	7.0	57	2	1
	0		High	6.9	1	98	0
	0		Low	7.2	68	2	4
Agstone	4	Oct. 22, 1930	High	6.3	1	98	0
	8	do	do	6.4	1	98	0
Calcium hydrate	1½	do	do	7.0	1	98	0
	1½	do	Low	7.3	49	16	8
	1½	Apr. 25, 1931	High	6.8	14	80	4
	1½	June 2, 1931	do	7.0	12	92	4
	2½	Oct. 22, 1930	do	7.0	1	98	0
	2½	do	Low	7.3	44	4	4
	2½	Apr. 25, 1931	do	7.2	57	4	8
	2½	June 2, 1931	do	7.2	52	0	0

The results of the field trials over a period of 3 years indicated that seasonal and soil factors influence greatly the action of liming materials as clubroot inhibitors. The remainder of this paper is concerned with the study of certain environmental factors in the greenhouse.

[9] CHUPP, C. See footnote 3.

GREENHOUSE EXPERIMENTS

In pot experiments in the greenhouse, Wellman [10] found that as the chemicals were added to the soil in increasing amounts, infection was inhibited by $Ca(OH)_2$ when the pH reached 7.3, by $CaCO_3$ when it reached 7.9, but when K_2CO_3 was applied until the pH reached 8.1 infection still occurred. This experiment was repeated by the writers and certain other chemicals were included. Untreated soil from the Miller field was used. The experiment was run in three replications in 2-gallon earthenware crocks. The soil moisture was held at 75 to 80 percent of the water-holding capacity. The results are given in table 4.

TABLE 4.—*Effect of application of various chemicals to clubroot-infested soil upon soil reaction and disease development in pots in the greenhouse*

Chemical applied	Rate of application per acre	Series 1			Series 2			Series 3		
		Soil reaction	Healthy plants	Diseased plants	Soil reaction	Healthy plants	Diseased plants	Soil reaction	Healthy plants	Diseased plants
	Tons	*pH*	*Number*	*Number*	*pH*	*Number*	*Number*	*pH*	*Number*	*Number*
Commercial Agstone	1	5.7	0	20	5.8	0	20	5.7	0	20
	2	6.9	3	17	7.0	5	15	6.9	4	16
	4	7.2	20	0	7.2	20	0	7.3	20	0
	8	7.6	20	0	7.5	20	0	7.7	20	0
CaCO₃	1	5.5	0	20	5.6	0	20	5.9	0	20
	2	6.9	13	7	7.0	8	12	7.0	9	11
	3	7.0	20	0	7.3	20	0	7.2	20	0
MgCO₃	1	6.7	4	16	6.9	6	14	6.9	8	12
	2	7.1	18	2	7.3	17	3	7.2	20	0
	3	7.3	20	0	7.3	20	0	7.2	20	0
Commercial calcium hydrate	1	6.9	3	17	7.1	4	16	7.0	6	14
	2	7.2	20	0	7.4	20	0	7.3	20	0
	3	7.4	20	0	7.4	20	0	7.6	20	0
Ca(OH)₂	1	6.8	2	18	7.1	4	16	7.0	5	15
	2	7.2	20	0	7.3	20	0	7.3	20	0
	3	7.6	20	0	7.4	20	0	7.3	20	0
CaO	1	7.1	20	0	7.1	20	0	7.2	20	0
	2	7.3	20	0	7.3	20	0	7.3	20	0
	3	7.8	20	0	7.4	20	0	7.4	20	0
Na₂CO₃	1	5.6	0	20	5.8	0	20	5.8	0	20
	2	5.9	0	20	6.1	0	20	6.1	0	20
	3	6.1	0	20	6.4	0	20	6.3	0	20
K₂CO₃	1	5.7	0	20	5.7	0	20	5.9	0	20
	2	5.9	0	20	5.8	0	20	6.1	0	20
	3	6.1	0	20	6.0	0	20	6.3	0	20
Na₂SO₄	1	5.8	0	20	5.6	0	20	5.6	0	20
	2	6.1	0	20	6.0	0	20	5.8	0	20
	3	6.4	0	20	6.1	0	20	6.1	0	20
Untreated soil	0	5.7	0	40	5.6	0	40	5.6	0	40

Na_2CO_3, K_2CO_3, and Na_2SO_4, in the amounts used, raised the pH only slightly and did not affect clubroot infection. Commercial Agstone, finely ground dolomitic limestone, distinctly inhibited infection when the soil reaction reached pH 6.9 and completely prevented infection at pH 7.2 and 7.6. $CaCO_3$ gave similar results, while $MgCO_3$ was not completely effective until the soil reaction reached pH 7.3. These results differ from those of Wellman, who did not secure complete inhibition with $CaCO_3$ until the pH reached 7.9. $Ca(OH)_2$ produced little effect at pH 6.8 and 6.9 but was completely effective at 7.2 and above, while CaO prevented infection at 7.1.

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[10] WELLMAN, F. L. See footnote 2.

It may be seen in this experiment that in the greenhouse those chemicals that were applied in amounts sufficient to raise the soil reaction slightly above neutral were in general very effective in preventing infection. This is in direct contrast to the results of field experiments described above in which the application of some of the same materials in quantities which raised the pH to 7.0 to 7.8 usually failed to prevent an abundant development of disease. As was pointed out earlier, in the Miller plot in 1930 and in the Franksville plot in 1932, calcium hydrate was effective in low portions of the fields where the soil moisture was relatively higher and conditions for plant growth were more favorable. In the greenhouse experiments a reasonably constant and favorable soil moisture was maintained and here also the liming materials were effective.

In the autumn of 1930 soil was secured from the untreated area and from each of the areas which had received 2- and 4-ton applications of calcium hydrate in the 1930 trials on the Miller plot. The reaction of the untreated soil was pH 5.8, that of the soil from the 2-ton area was pH 7.1, and that from the 4-ton area was pH 7.4. The three lots of soil were placed in 2-gallon earthenware jars and kept at 70 to 80 percent of the water-holding capacity. Twenty plants were grown in each sample for 1 month and the experiment was replicated at three different times. Heavy infection with clubroot was secured throughout the series in every plant on the untreated soil. No infection whatever occurred in the soil from the treated areas. In the autumn of 1931 soil was collected from the areas of the Miller plot which had received 4-ton and 8-ton applications of Agstone in October 1930. It may be recalled that in the 1931 season there had been no evidence of disease inhibition in these areas (table 1). At the time of collection the soil from the 4-ton Agstone area had a reaction of pH 7.4, and that from the 8-ton area had a reaction of pH 7.8. This soil was set up as described in the last experiment, and untreated soil was again used as a control. Forty plants were grown in each sample of soil for approximately 4 weeks. At the end of that time all plants in the untreated soil were severely diseased, none was diseased in the soil receiving the 8-ton treatment, while in that receiving the 4-ton treatment about 50 percent of the plants were infected. The experiment was repeated and the outcome was similar. These results gave conclusive evidence that the conditions surrounding the experiment in the greenhouse permitted complete control in the soil treated with 2-ton and 4-ton applications of calcium hydrate and an 8-ton application of Agstone, while conditions in the field had allowed heavy infection in the same soils. These results conform with those of other workers, who have secured consistent and convincing evidence of the effectiveness of lime in greenhouse tests while in the field various degrees of inconsistency have prevailed.

A study of this soil was continued by taking samples from the calcium-hydrate-treated areas over a period of about 2 years, and conducting greenhouse trials in the manner described above. The results are given in table 5. It is to be noted that as the interval after treatment increased the reaction of the treated soil gradually reverted to neutral and below. As the soil became more acid the percentage of infected plants gradually increased.

TABLE 5.—*Clubroot infection in field-treated soil when removed to the greenhouse at various intervals after treatment*

Treatment	Greenhouse tests with soil collected after—											
	2 months			12 months			17 months			23 months		
	pH	Plants used	Plants infected	pH	Plants used	Plants infected	pH	Plants used	Plants infected	pH	Plants used	Plants infected
		Number	*Percent*		*Number*	*Percent*		*Number*	*Percent*		*Number*	*Percent*
Calcium hydrate, 2 tons per acre	7.1	70	0	7.0	70	18	6.8	70	68	6.4	70	90
Calcium hydrate, 4 tons per acre	7.4	70	0	7.3	70	0	7.0	70	0	6.7	70	40
Dolomitic hydrate, 2 tons per acre	7.2	70	0	7.1	70	12	6.9	70	56	6.4	70	70
Dolomitic hydrate, 4 tons per acre	7.6	70	0	7.4	70	0	7.1	70	0	6.9	70	30
Untreated	5.8	70	100	5.8	70	100	5.6	70	100	5.6	70	100

In the 1930 and 1931 field trials the soil moisture was relatively low for a major portion of the time, while in the greenhouse experiments it was kept consistently high. In order to ascertain whether soil moisture was a determining factor in the effectiveness of lime, soil from both 2-ton treatments with calcium and dolomitic hydrate in the 1930 Miller plot was set up at fairly constant soil-moisture levels of about 40 to 50 percent, 55 to 65 percent, and 70 to 80 percent of the water-holding capacity. Plants were transplanted to the crocks and the moisture levels maintained for 5 weeks. At the end of that time the plants in the untreated soil were uniformly infected at all moisture levels. In treated soils there was complete inhibition of infection at all moisture levels. The experiment was extended to the 8-ton Agstone-treated soil from the 1931 Miller plot. This soil was held at moisture levels of 40 to 50, 50 to 60, 60 to 70, 70 to 80, and 80 to 90 percent of the water-holding capacity. After 4 weeks all plants on the untreated soil held at 70 to 80 percent of the water-holding capacity were diseased. There were no diseased plants in the Agstone-treated soil at any of the moisture levels. The entire experiment was repeated with identical results.

Although the effectiveness of the two types of hydrate and Agstone as inhibitors of infection was uniform over a range of relatively constant soil-moisture levels, the conditions which prevailed still did not simulate those in the field, where there is usually a fluctuation of the moisture content of the soil. The next experiment was set up to observe the effect on infection of fluctuation in soil moisture.

Untreated soil and soil from the 2-ton treatment with calcium hydrate in the 1930 Miller plot were used in the winter of 1930–31. Two earthenware crocks were filled with the calcium hydrate field-treated soil and two with untreated soil. Ten plants were set in each crock and the moisture content of the soil was held at 70 to 80 percent of the water-holding capacity. Another lot of the treated soil was allowed to dry out until the moisture content was below 16 percent of the water-holding capacity. Four flats 20 inches long, 14 inches wide, and 12 inches deep were filled to a depth of 9 inches. Six

plants were set in each flat, each plant being watered in with 30 cc of water. After 5 days 50 cc of water was added to each flat by spraying it uniformly over the surface. After another 5 days 100 cc was added in the same manner and this procedure was repeated every 5 days during the remainder of the 6 weeks' duration of the experiment. Thus, in the earthenware crocks the soil moisture was quite constant and relatively high throughout the experiment. In the flats, however, the conditions resembled more closely those in the field trials, where plants were watered into relatively dry soil. The high moisture content of the soil around such transplants, besides being sufficient for infection, stimulated emergence of new secondary roots, which soon grew into the soil. The moisture content of this soil varied considerably from day to day and at different distances from the surface.

After 6 weeks the plants from all crocks and flats were removed and the experiment was repeated. The results of both series are given in table 6. In the crocks in which the moisture content was kept reasonably constant the results of previous experiments were duplicated. Every plant in the untreated soil was infected, but there was no infection in the treated soil. On the other hand, in the flats in which the moisture around the plants was relatively high at the time of transplanting, but was allowed to fluctuate in the whole bulk of the soil, every plant was infected. It is true that the average soil moisture in the flats was lower than in the same soil in the crocks, but infection cannot be attributed to low soil moisture alone since in previous experiments constant low soil moisture failed to favor infection. It appears that the fluctuation of soil moisture at relatively lower levels provided the proper conditions for infection in spite of the fact that calcium hydrate had been incorporated in the soil. Under these conditions the organism infected the plants readily as it had done earlier in the 1930 Miller plot.

TABLE 6.—*Clubroot infection in calcium-hydrate-treated and in untreated soil at constant and fluctuating soil moisture*

Treatment	Condition of soil moisture	Series 1		Series 2	
		Plants used	Plants infected	Plants used	Plants infected
		Number	*Percent*	*Number*	*Percent*
Untreated	Held at 70 to 80 percent of the water-holding capacity in crocks.	20	100	20	100
Calcium hydrate, 2 tons per acre.	___do___	20	0	20	0
Do	Started at 16 percent of the water-holding capacity, and allowed to fluctuate in flats.	24	100	24	100

Similar lots of treated and untreated soil were next set up in 2-gallon earthenware crocks at 70 to 80 percent of the water-holding capacity. The untreated soil and part of the crocks containing treated soil were held at constant high soil moisture by daily weighing and addition of water. The remaining crocks of treated soil were allowed to dry out gradually until the soil moisture approached 40 percent of the water-holding capacity. They were then brought up to the original moisture content by the addition of water. This

process was repeated twice during an interval of 4 weeks. When the plants were removed and examined all those in the untreated soil were diseased. In the treated soil no infection occurred where the moisture was held constant nor where the moisture level fell and rose periodically. Thus this type of fluctuation had the opposite effect from that in which fluctuation at low soil moisture occurred.

Another experiment was conducted to determine the effect of aeration of treated soil upon infection. Two-ton calcium-hydrate-treated soil was placed in 2-gallon earthenware crocks and a perforated soft lead tube was incorporated in the soil. By this means a continuous flow of air was forced through the soil. An equal number of nonaerated crocks were used. During the course of the experiment the soil moisture fluctuated from 65 to 80 percent of the water-holding capacity. After 30 days the plants were removed and the roots examined. The results of two series of experiments, given in table 7, show that the introduction of air into the treated soil resulted in 35 to 40 percent infection, while in nonaerated treated soil only 1 plant in 40 became infected. In untreated nonaerated soil all plants were severely diseased.

TABLE 7.—*Effect of aeration of calcium-hydrate-treated soil upon clubroot infection*

Soil treatment	Aeration	Series 1		Series 2	
		Plants used	Plants infected	Plants used	Plants infected
		Number	*Percent*	*Number*	*Percent*
Calcium hydrate, 2 tons per acre.	{ Aerated	20	35	20	40
	{ Nonaerated	20	5	20	0
Untreated	do	20	100	20	100

DISCUSSION

Under the climatic conditions of 1930 to 1932, inclusive, hydrated lime did not uniformly inhibit clubroot infection in the field, although the soil reaction was in many cases changed to pH 7.0 or higher. However, when the same soil was removed to the greenhouse and plants were grown in it, the treatment was completely effective in preventing infection. Under the latter condition, where frequent watering was carried out, calcium hydrate, calcium carbonate, calcium oxide, and magnesium carbonate all reduced infection perceptibly when added in amounts sufficient to raise the pH to about 7.0, and usually inhibited infection completely at pH 7.2 or above. High or low relatively constant soil moisture did not change the degree of inhibition. Fluctuation of soil moisture and forced aeration, however, did permit varying degrees of infection in soil. It appears that fluctuation in soil moisture is likely to permit infection in slightly alkaline field soils.

While this study does not explain the fundamental bases of the effectiveness of lime as a clubroot inhibitor under various conditions, the results do show significant differences between greenhouse and field trials. It is obvious that greenhouse pot tests are not a true index of what may be expected in the field. If one were to consider

the greenhouse results given in table 4 alone there is ground for concluding that a distinct correlation exists between soil reaction and infection by the clubroot organism. Why this does not hold in the field is not explained, but it is clear that when the soil moisture fluctuates at relatively low levels abundant infection may occur at soil-reaction values which give no infection under the conditions of the experiments recorded in table 4.

It is suggested in this connection that the soil-reaction determination as ordinarily made is not necessarily a true index of the actual pH value of the soil closely adjacent to the roots. Thom and Humfeld [11] studied the reaction of soil particles adherent to fibrous root from a number of crop plants grown in a range of acid and alkaline soils. The soil in this zone, compared with the soil mass as a whole, was less acid when an acid soil was used, and less alkaline when an alkaline soil was used. It is possible that in the slightly alkaline treated soil employed in this study, the reaction in the immediate vicinity of the roots was sufficiently acid, under field conditions, to permit abundant infection. It is evident that treatment of soil sufficient to inhibit infection in the greenhouse in no sense eliminates the organism. When the reaction of such a soil naturally reverts to below pH 7.0 abundant infection occurs even when favorable soil moisture is maintained (table 5).

These results are offered only as they apply to the two soil types used. It is quite possible that heavier soils, higher in water-holding capacity, would yield entirely different results.·

SUMMARY

Field treatments of Carrington silty clay loam and Clyde silty clay loam with calcium hydrate and calcium magnesium carbonate which raised the reaction to pH 7.1 and above did not generally inhibit clubroot development.

In treated soils, removed to the greenhouse, cabbage plants remained free from infection while in untreated soil from the same field there was abundant disease development.

Under greenhouse conditions infection was inhibited in treated soil at high, intermediate, and low relatively constant moisture levels.

Calcium hydrate, calcium carbonate, calcium oxide, and magnesium carbonate all reduced infection perceptibly in well-watered soil when added in sufficient amounts to raise the reaction of an acid soil to about pH 7.0, and usually inhibited infection completely at pH 7.2 or above.

Fluctuation of soil moisture at a relatively low soil-moisture level and forced aeration of the soil permitted varying degrees of infection in treated soils.

It is suggested that low fluctuating soil moisture is an influential factor in limiting the effectiveness of lime as a clubroot inhibitor in the field.

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[11] Thom, C., and Humfeld, H. Notes on the association of microorganisms and roots. Soil Sci. 32: 29-36. 1932.

LIFE HISTORY OF THE CROWN-GALL ORGANISM IN RELATION TO ITS PATHOGENESIS ON THE RED RASPBERRY [1]

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INTRODUCTION

Crown gall, caused by *Phytomonas tumefaciens* (Smith and Town.) Bergey et al. (*Bacterium tumefaciens* Smith and Town.), is a serious and wide-spread disease of cane fruits. Though much attention has been given to various other phases of the crown-gall problem, comparatively little study has been made of it on cane fruits, and the life history of the causal organism in relation to pathogenesis has been but imperfectly understood. The present investigation was undertaken in an effort to define important points in the life history of the causal organism in relation to its pathogenesis on the red raspberry (*Rubus strigosus* Michx.) in the hope that such knowledge might contribute toward the development of control measures.

One strain of *Phytomonas tumefaciens* was used in most of the experiments reported herein. This strain was originally isolated by the writer from a crown gall on Wealthy apple. It has been described as strain 2018 in studies recently reported by Riker et al. (*27, 28*),[3] and was repeatedly demonstrated throughout the entire course of these investigations to be highly pathogenic on tomato, *Sedum*, tobacco, apple, *Bryophyllum*, sugar beet, and the underground parts of both red and black raspberry varieties. The reactions on several common laboratory media and host plants, induced by 18 strains of the crown-gall organism isolated by the writer from as many collections of crown gall on the roots of red raspberry from Wisconsin, Indiana, and Michigan, have not differed from those induced by strain 2018.

EXIT OF THE CROWN-GALL ORGANISM FROM THE HOST

It has frequently been considered that the crown-gall organism is released from the galls only at the time of their disintegration. From the recent experimental work discussed herein it has been found, however, that under favorable moisture conditions crown-gall bacteria are continuously passed off from the surface of living crown galls.

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That the soil in which diseased plants are grown contains the crown-gall organism has been suggested by several investigators, who have reported that susceptible plants grown in such soil become diseased (*1, 2, 13, 17, 21, 25, 34, 35, 38*). Patel (*22*) has reported successful isolation of the crown-gall organism from the soil of nursery fields that had produced crops affected by the disease. It has commonly been assumed that the bacteria in the galls are liberated when the galls decay (*5, 6, 17, 18*). The limited experimental evidence does not sustain this widely held assumption. Riker and Keitt (*29*) reported that "from two dozen recorded attempts at isolation from decaying crown-gall tissue, no crown-gall bacteria were found." The present writer has likewise been unsuccessful with either potato-dextrose or bile-agar media (*22*) in many isolation trials from decaying or decayed crown-gall tissue. Robinson and Walkden (*31*) reported that they had observed great numbers of crown-gall bacteria on the surface of living crown galls and that prolonged exposure to running water reduced the bacterial population on the surface by about 200 to 1.

Each of two active crown galls free from decay was successively immersed for different periods in a series of equal volumes of sterile water (table 1). Upon transfer of the gall from one quantity of water to the next in the series, a bacterial plate count was made of the water in which the gall had just been immersed. The results (table 1) are from (1) a crown gall induced on the underground portions of a tomato stem by inoculation about 30 days prior to the time of the experiment, and (2) a crown gall induced by inoculation on the underground portion of the scion of a vigorously growing Wealthy apple graft about 10 weeks prior to the time of the experiment. The bacterial counts made of the successive wash waters show (1) that during the time of the experiment the bacteria were passing from the surface of the gall to the surrounding water, and (2) that the number of bacteria given off to the water during any immersion period was a very small fraction of the total bacterial population of the gall.

TABLE 1.—*Number of crown-gall bacteria found in successive 10 cc volumes of sterile water in which a crown gall had been immersed for the time indicated in the sequence given*

Specimen and treatment	Duration of treatment	Bacteria found in water	Specimen and treatment	Duration of treatment	Bacteria found in water
Tomato crown gall:	*Minutes*	*Number*	Apple crown gall—contd.	*Minutes*	*Number*
Immersed in water [a]	10	4, 050, 000	Immersed in water [a]	10	2, 120, 000
Do	30	4, 800, 000	Do	40	49, 670, 000
Do	10	697, 000	Do	10	893, 400
Do	10	2, 717, 000	Surface washed in running water [c]	70	
Do	30	13, 470, 000			
Do. [b]	10	1, 867, 000, 000	Immersed in water [a]	10	264, 120
Apple crown gall:			Do	50	19, 463, 000
Immersed in water [a]	10	3, 267, 000	Do. [b]	10	550, 000, 000
Surface washed in running water [c]	35				

[a] Immersed in 10 cc of sterile water in a large test tube.
[b] Macerated and placed in 10 cc of sterile water.
[c] Placed on cheesecloth in a stream of water falling from a faucet.

Aerial portions of the stems of a number of succulent tomato plants were sealed in small cylindrical glass chambers within which the bacterial flora could be controlled. These cylinders were 1¼ inches in

diameter by 3 inches long, with a T side-arm extension of glass tubing one half inch in diameter and three fourths of an inch long. They were slipped down over the leaves of young tomato plants and held in place by clamps supported on ring stands. The open ends of the cylinders were closed by thin sections of a one-hole rubber stopper of suitable diameter. Adhesive tape was bound over the outside of the stoppers and the ends of the cylinders. The ends were then sealed with a mixture of vaseline and paraffin, which was poured in a liquid state through the side arm and around the stem at the bottom of the cylinder. After the mixture had hardened, a thin layer of liquid vaseline was placed on top of the paraffin-vaseline mixture. The top end of the cylinder was sealed by holding the potted plant with the attached cylinder upside down. All motion of the stem within the cylinder that might break the vaseline seal in subsequent manipulations was prevented by binding the pot in which the plant was growing to the base of the ring stand supporting the cylinder (fig. 1).

The surfaces of those portions of the stems which were sealed in the cylinders were then disinfected, inoculated, and again disinfected. Disinfection was accomplished by filling the cylinders for 10 minutes with silver nitrate solution (1: 1,000), introduced, as were all materials subsequently employed, through the side arm of the cylinder, the free end of which was closed by a removable rubber stopper.

FIGURE 1.—Glass cylinder sealed over a section of tomato stem. In this manner sections of tomato stem were held for short intervals of time under aseptic conditions.

After the silver nitrate solution had been removed, the cylinders were washed with three successive changes of sterile water, each of which was held in the chamber for 10 minutes.

A plate count was made of a fourth change of sterile water as a check on the effectiveness of the disinfection. One day after disinfection the sterile surfaces of the stems within the cylinders were inoculated by thrusting into each stem a needle that had been smeared

with a crown-gall culture. As protection against contamination, cheesecloth moistened with silver nitrate solution was held over the cylinder during this and other manipulations. Two days after inoculation the stem surfaces within the cylinders were again disinfected, and a plate count similar to that just described was made as a check on the effectiveness of the disinfection.

Two months later an estimate was made of the number of crown-gall bacteria on the surfaces of the stems and large galls induced within the chambers by inoculation. This estimate was obtained by plate counts made of sterile water with which the chamber had been filled and in which the gall had been immersed for 10 minutes. The results from three cylinders in which galls had been successfully induced by the method just described showed that immediately after the first and the second disinfections no crown-gall bacteria were present on the surface of the stem. Moreover, no bacterial or fungal colonies developed in these plates. But 2 months later the plate counts showed 1,000,000, 4,000,000, and 24,000,000 crown-gall bacteria present on the surface of the stems and galls enclosed, respectively, by the three cylinders.

Before disinfection of the gall surfaces, immediately after disinfection, and at various intervals of time thereafter, estimates were made of the bacterial population on the surfaces of three other tomato crown galls held under aseptic conditions within the glass cylinders just described. Before disinfection an average of 3,750,000 living crown-gall bacteria were found. Thirty minutes after disinfection there was none; 4 hours later there were 4,000; 36 hours after disinfection, 13,500; and 5 days after disinfection, 4,400,000.

LONGEVITY AND OVERWINTERING IN SOIL

Observations over a number of years have led to the belief that the crown-gall organism may live for some time in soil in which diseased plants have grown (*1, 2, 13, 21, 34, 35*). Experimental evidence reported herein demonstrates that the crown-gall organism overwinters in the soil under ordinary field conditions and that it may live for more than a year in unsterilized soils held free from the growth of seed plants.

Experimental evidence as to the overwintering and longevity of the crown-gall organism has been reported by several writers (*3, 19, 23, 24*). Muncie (*19*) states that tomato plants transplanted to inoculated soil became infected until 154 days after the soil had been inoculated. Patel (*23*) reported that he was able to recover the organism in a pathogenic state from inoculated unsterilized soils in which it had overwintered under field conditions at Ames, Iowa. From a series of isolation trials made from various types of inoculated unsterilized soils held moist under laboratory conditions, he concluded that the organism "may live longer in sandy soils than in clays." He was able successfully to isolate and demonstrate the pathogenicity of the organism 669 days after inoculation in unsterilized loam. On the basis of isolations from the soils of a number of nursery fields, Patel reported that "the pathogenic form of *Ps. tumefaciens* is localized in the field, * * * in close proximity to true galls", and that "organisms resembling

Ps. tumefaciens are not generally found in soils from which crops susceptible to this pathogene are absent."

To determine whether the organism overwinters under field conditions at Madison, Wis., two plots of soil 3 feet square were inoculated September 23, 1926, and isolations were attempted in November and again in May of the following spring. Two diverse soil types were chosen, one a neutral peat and the other a Clyde silt loam. Inoculation to a depth of 2 feet was accomplished by removing the top layer of soil and treating the underlying layers first with a heavy suspension of *Phytomonas tumefaciens.* This was prepared by diluting, at the rate of 1 culture per 6 quarts of water, 6-day mass cultures grown on agar slopes in flat 11-ounce bottles. The suspension was mixed at once with the soil at the rate of 1 pint per cubic foot of soil. Soil samples for isolation were taken from 4 to 7 inches below the surface of the soil in each plot. Soil dilutions of 1:1,000, 1:10,000 and 1:100,000 were used to pour agar plates with Patel's bile medium (*22*). Five plates were poured for each dilution and were incubated 6 days at 28° C. They were then examined for crown-gall-like colonies, a number of which were selected, and with each colony from 2 to 10 inoculations were made on tomato stems. The ability of these bacteria to induce galls when inoculated into susceptible tomato stems was the criterion used in identifying the crown-gall organism. The results, recorded in table 2, show that the organism overwintered in the soil of these two fallow field plots.

TABLE 2.—*Overwintering of Phytomonas tumefaciens in 2 types of soil under field conditions at Madison, Wis., 1926–27* [a]

Date of isolation	P. tumefaciens from neutral peat soil			P. tumefaciens from Clyde silt-loam soil		
	Colonies tested	Total inoculations	Inoculations positive	Colonies tested	Total inoculations	Inoculations positive
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
Nov. 12, 1926	20	40	85	20	40	95
May 21, 1927	17	68	68	34	136	15

[a] The soils were inoculated on Sept. 23, 1926.

To gain information as to the length of time the organism might exist in the soil in a pathogenic state, isolations were attempted periodically from two samples of unsterilized inoculated soil. The types used were the neutral-peat and silt-loam soils referred to above. They were held moist and free from the growth of seed plants in open cans in a cellar used for the storage of nursery stock. The isolation technic used and the criterion of successful isolation were the same as in the overwintering studies. The degree of success was measured by the percentages of the inoculations made with these colonies that induced crown galls on tomato stems. From the data recorded in table 3, it is clear that the organism can live for over a year in a pathogenic state in the soil in the absence of seed plants.

When inoculated into tomato stems, the last crown-gall colonies isolated from the silt-loam soil induced but very small galls. Four

TABLE 3.—*Longevity of Phytomonas tumefaciens in samples of 2 unsterilized soils kept in a storage cellar at Madison, Wis., 1926–27* [a]

Date of isolation	*P. tumefaciens* from neutral peat soil			*P. tumefaciens* from Clyde silt-loam soil		
	Colonies tested	Total inocula-tions	Inocula-tions positive	Colonies tested	Total inocula-tions	Inocula-tions positive
1926	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*
May 14	10	20	100	10	20	100
May 31	10	20	100	10	20	100
June 11	10	20	100	10	20	100
June 24	10	20	100	10	20	100
July 25	10	20	100	10	20	80
Sept. 10	10	20	100	10	20	0
Nov. 7	10	20	100	10	20	70
Dec. 7	5	15	100	3	24	29
1927						
Mar. 3	10	40	80	4	24	25
July 2	10	40	70	6	36	0
Oct. 10	5	20	0	1	8	0
Nov. 15	5	20	0			

[a] The soils were inoculated on May 1, 1926, with pure cultures and held under approximately constant moisture conditions at 17° C.

inoculations were made into each of two tomato stems with the three crown-gall-like colonies isolated December 7, 1926. All the inoculations made with two of these colonies were negative. Seven of the eight inoculations made with the third colony produced uniform galls that were, however, very small as compared with the galls induced by parallel inoculations from a 6-day-old transfer of the stock culture with which the soils had been inoculated. Correspondingly, one of the four colonies isolated March 3, 1927, induced relatively small overgrowths on tomato. Although other crown-gall-like colonies were isolated in these and in subsequent trials, none induced any overgrowth response whatsoever in similar pathogenicity tests. These results suggest that the ability of the organism to induce galls on tomato may have declined after it had lived for a long period in the soil; or the small size of the galls induced by the last colonies isolated may have been due to competition with other organisms contained within these colonies (*7, 27, 43*).

DISSEMINATION

That crown gall is spread to new areas by the shipment of infected nursery stock has long been known, and careful inspection has often been relied upon to prevent this spread. The writer's experimental evidence discussed herein indicates that rigid inspection of raspberry plants does not prevent the disease from being spread in this way, because incipient stages of infection, which no amount of careful inspection can detect, may be carried by apparently healthy plants.

Selby (*33, pp. 111–112*) reported the disease to have occurred in a number of raspberry plantations in the vicinity of Florence, Ohio, and stated that "* * * all the growers procured the plants from the same source. The plantation from which these plants came was found, on examination, to be diseased in the same manner." Lawrence

(*17, p. 17*) 10 years later warned Washington growers against using plants from diseased fields, and said:

* * * even if only those plants are used which do not show signs of the galls the trouble will be quite sure to manifest itself at a later period. The writer has seen this occur in a number of instances.

Bennett (*6, p. 22*) reported:

Plants which have been heeled in or which have been held in cold storage and wet down several times, although they may have few visible galls when planted in the field, have been known to become severely diseased often before the end of the first season. Apparently, in such cases, the bacteria are washed from the few galls which may be in the bundles and find their way into wounds where they become established but do not produce visible swellings till after planting.

Investigators who have studied crown gall have usually found it difficult to obtain plants free from the disease. Thus Smith et al. (*39*) could draw no conclusions from certain inoculation experiments on red raspberry because of the infection that appeared on the roots of the uninoculated control plants. They state that "the infection was probably brought along with them from the nursery, because one or two knots were found on the roots of these plants when they were purchased." Colby's experience, as reported by Keilholz (*16*), in conducting experiments on control of crown gall on raspberries by means of germicides has been similar. He states that conflicting results come

* * * from not knowing whether the plants were clean when planted. An attempt was made to set only clean plants, but it is impossible to detect the crown-gall infection in its incipient stage. If the plant is already infected at planting time, the infection is quite certain to enlarge unless the soil near the plant has been so heavily treated with the germicide that the plant itself is killed.

A test was made by the writer to determine whether apparently healthy red raspberry plants carry incipient stages of infection. Plants of the Latham variety were obtained from a nursery in which the disease was known to be present. These plants were carefully examined for macroscopically visible infections before planting. A number of such plants were found bearing galls 1 mm or more in diameter. These were discarded. Plants selected as not visibly diseased were completely submerged for 20 minutes in a silver nitrate solution (1:1,000) to prevent contamination by crown-gall bacteria that might be present on the surface. They were then set out in beds of soil which had been steamed.

These beds had been heated by the inverted-pan method of high-pressure steaming described by Johnson (*15*). Each bed after being steamed for 30 minutes with a head of 120 pounds pressure, was surrounded by boards as protection against contamination by surface water and left for 1 week before planting. Ten beds thus prepared were planted May 12, 1928, with 600 raspberry plants that had been selected and treated as described.

Twenty-five to fifty raspberry plants were dug and examined at intervals of 2, 3, 11, 12, and 18 months after planting. Of 344 plants harvested from these steamed beds during this period, 51, or 15 percent, were affected with crown gall. An average of 1.7 infections was found on diseased plants harvested within 1 year after planting. The size and condition of the galls found on plants during the first growing season indicated that infection had occurred prior to or soon

after the time of planting. Since it seems improbable that crown-gall organisms in the soil were not killed by the steaming or that the soil was reinfected during the first growing season, it appears that the galls which developed on these plants were due to incipient infections which were present on the plants when received from the nursery but which were not visible. This view is supported by further evidence reported in a later section dealing with (1) the protracted incubation period of crown-gall infections under unfavorable growing conditions and (2) the length of time wounds remain potential avenues of infection by *Phytomonas tumefaciens*. On the raspberry, the disease has been reported by Bennett (*6*) to occur through wounds, and by Dodge and Wilcox (*9*) "through wounds made by insects or by mechanical injuries." The Great Britain Ministry of Agriculture and Fisheries (*12*) very succinctly summarizes the literature on this subject by saying:

It is generally believed that infection can occur only through wounds, for practically all attempts to produce Crown Gall by inoculation of unwounded tissues have given negative results, whilst the frequent occurrence of Crown Galls on parts of plants which have been cut or wounded strongly supports the view that the organism is a wound parasite. The fact that Crown Galls occur on roots and occasionally on seedlings where no evidence of wounding is apparent does not negative this view, for insignificant wounds made by soil insects or by other means would suffice as ports of entry for the organism.

Observations and experiments have been made by the writer, as previously reported in abstract form (*4*), to determine (1) whether injuries on the underground parts of the red raspberry remain subject to infection for relatively long periods, (2) whether in the absence of injuries to the underground parts of the plant crown-gall infection may occur, and (3) whether root-feeding arthropods are chiefly responsible for the injuries through which crown-gall bacteria gain entrance to the tissues of the plant. The details of this work follow.

LENGTH OF TIME INJURIES ON HOST REMAIN OPEN TO INFECTION

The pronounced susceptibility of the red raspberry to crown-gall infection might be explained in some measure were it demonstrated that injuries on the underground parts of this plant remain open to crown-gall infection for relatively long periods. This phase of the problem was studied in four series of experiments over a period of 3 years. Plants in various stages of development were studied under both field and greenhouse conditions. In both types of experiment all injuries were made at one time and inoculum was applied at various intervals after wounding. The injuries in all cases were made by scraping through the cambial layer with a scalpel. The plants used were grown in soil that had previously been steamed, and precautions were taken against contamination of the injuries by crown-gall bacteria before the inoculum was applied. In experiments on plants in the field, because of the comparative inaccessibility of the roots, most of the injuries were made on the underground portion of the stem. After the soil around the points of injury had been removed, the inoculum was applied to the injuries by means of a camel's-hair brush. Every precaution was taken not to disturb the tissues at the points of injury when the soil was removed and the inoculum applied. Examination of the wounds was made several months after the inoculum was applied to the last set of injuries in the

series.   The results of the field studies made in this manner (table 4) indicate that injuries on the underground parts of the red raspberry in some cases may remain open to invasion by the crown-gall organism for 6 weeks.

TABLE 4.—*Length of time during which crown-gall infection was secured through injuries made on stems of red raspberry plants in the opening-bud stage of early spring[a] and in the early autumn[b]*

OPENING-BUD STAGE OF EARLY SPRING

Period between injury and inoculation (days)	Plants injured	Injuries inoculated[c]	Injuries infected at close of season
	Number	*Number*	*Percent*
1	7	28	57.1
2	5	20	70.0
6	6	24	54.2
18	7	28	43.0
Controls	15	60	0

EARLY AUTUMN

2	21	72	77.8
5	17	66	57.6
9	13	43	16.3
42	[d]13	44	15.2
Controls	20	60	1.6

[a] The injuries were made on the underground section of the stems of red raspberry plants, just before planting them in steamed soil on May 8, 1928.
[b] The injuries were made Sept. 1, 1928, on the underground sections of the stems of red raspberry plants grown in steamed soil.
[c] A suspension of crown-gall bacteria was applied to the injuries with a camel's-hair brush at various intervals after the time of injury.
[d] These plants were practically in a dormant condition when the inoculum was applied to the injuries. The results were recorded at the close of the following season.

ENTRANCE INTO HOST

"Everything we know about crown gall points to wounds as the usual, if not the only way of infection" (*38*).   This view, held by Smith in 1920, has been supported by the observations (*10, 11, 17, 19, 29, 32, 41, 42*) and experiments of all later investigators.[4]   Infection has been obtained experimentally on apple (*19, 20, 27, 29, 36, 37*), tomato (*19, 26*), *Ricinus* (*26, 30*), geranium (*30*), and grape (*17, 18*) only through wounds.[5]

Under greenhouse conditions similar studies were made on the roots of red raspberry plants grown in root-observation boxes.   These plants had been grown for 1 year in the steamed-soil plots previously described and were free from crown-gall infections when set out in the boxes in the spring, since those that carried latent infections from the nursery had been detected and discarded.   The soil in the root-observation boxes was steamed at the beginning of the experiment.   In August injuries were made on the roots in the manner already described, and at various periods of time thereafter inoculum was applied to them.   Several months after the last group of injuries in

[4] A possible exception is Stapp (*40*), who reported that tumors caused by *Bacterium* (*Phytomonas*) *tumefaciens* occurred at the eyes of potatoes that were planted either in inoculated soil or in ordinary garden soil after being dipped in a suspension of the organism.
[5] Experiments made by various investigators with *Phytomonas rhizogenes* (*28*), often called the apple strain of the crown-gall bacterium, are not included, although recent research (*14*) on this organism shows that it gains entrance to the tissues of its host primarily if not solely through injuries.

the series was treated, all the injuries were examined and the presence or absence of the disease was recorded.

Root-observation boxes of a type similar to those described by Dean (8) were used in these experiments. The sides of the boxes were made of wooden hinged doors that covered removable glass plates. The wooden doors could be opened readily and through the glass plates the roots in contact with the glass at the surface of the soil could be observed without disturbing them. The glass plates were removed when the injuries and inoculations were made. The use of these boxes made it possible to manipulate and observe the roots without danger of causing further injury by removing the surrounding soil, as must be done in field experiments. By the middle
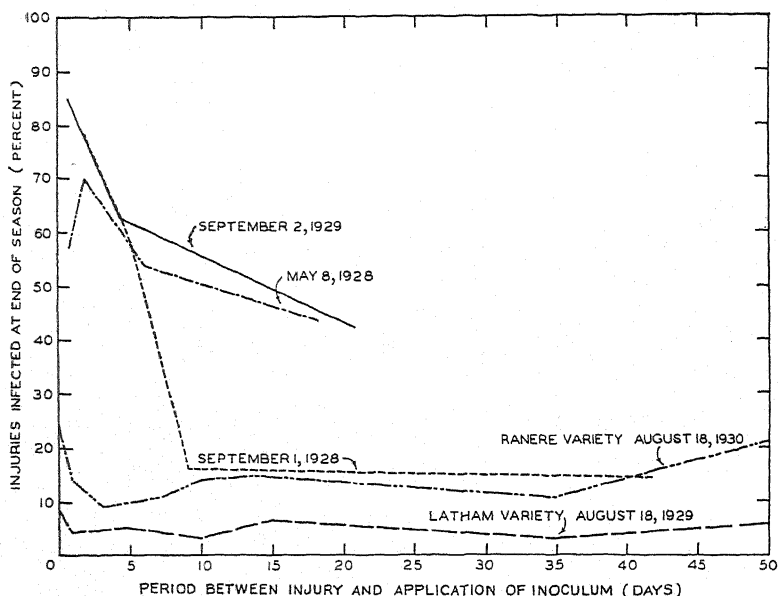
FIGURE 2.—Length of time during which crown-gall infection was secured through wounds on underground parts of red raspberry. The dates given are the dates at which the injuries studied in the experiments were made.

of August the roots in the boxes had grown extensively and a considerable number were in contact with the glass plates at the sides of the boxes.

Injuries were made with a scalpel, as in the field experiments. The injuries were then marked by means of map tacks in the ends of match sticks that were pushed into the soil so that the head of the map tack was adjacent to the injury at the surface of the soil. The injuries in each group in the series were made on the roots of plants in 1 or 2 boxes and inoculation of all the injuries in one group was made at one time, by sprinkling the roots on the surface of the soil with a suspension of crown-gall bacteria. Inoculated boxes were kept separate from those as yet uninoculated, to avoid the danger of contamination of the injuries with crown-gall bacteria before the desired time interval had elapsed. The results of the experiment are shown

in table 5. This experiment was repeated under like conditions the following season, and the time was extended to 50 days. Essentially the same result was obtained as in all earlier experiments (lowest curves, fig. 2).

TABLE 5.—*Length of time during which crown-gall infection was secured through injuries made on roots of red raspberry plants in early autumn,* [a] *1929*

Treatment and date	Period between injury and inoculation	Injuries treated	Injuries infected	
			Oct. 3	Nov. 26
Injuries inoculated: [b]	*Days*	*Number*	*Percent*	*Percent*
Sept. 3	1	40	75	85
Sept. 6	4	50	40	64
Sept. 23	21	50	0	42
No inoculum applied		100	0	0
Control injuries made and inoculated:				
Sept. 3	0	10	70	90
Sept. 6	0	10	60	90
Sept. 23	0	10	0	70
Oct. 4	0	31	[c] 10	71
Control needle-thrust inoculations:				
Aug. 22–Sept. 3	0	90	87	96
Oct. 4	0	31	[c] 7	77

[a] The injuries were made on Sept. 2 on the roots of healthy crown-gall-free plants grown in steamed soil in root-observation boxes.
[b] A suspension of crown-gall bacteria was sprayed over the root and the soil in the root-observation boxes.
[c] Observed on Oct. 24.

The data obtained from each experiment are presented in figure 2. The curves show that the percentage of treated injuries that became infected decreased as the interval between the time of injury and the application of inoculum increased. On vigorous plants this decrease was at first considerable, but after 10 days to 3 weeks the percentage of infected injuries tended to remain constant, indicating probably that a fair proportion of such injuries were rapidly closed to infection but that a small part of the injuries remained subject to infection for 3 to 7 weeks or longer.

A study was made to determine whether injuries on the underground parts of a variety more resistant to crown gall would be subject to invasion by the parasite for a shorter time. Latham and the supposedly more resistant variety Ranere were used. The experiment was carried out on each variety under the same conditions and in the same manner as the experiments described above. The results are recorded in figure 2. The erratic results obtained in the 1930 season may have been due in part to the low vitality of the plants used, a condition that probably resulted from late planting. The results indicate, as did those of all earlier experiments, that injuries to the underground portion of the Latham red raspberry remain subject to crown-gall infection for a considerable period. The comparative record on the more resistant variety, Ranere, suggests that in this respect there is little difference in these red raspberry varieties.

Ranere, often called St. Regis, has been reported resistant and Cuthbert susceptible to crown gall. Comparative studies by the present writer of the development of crown gall in five varieties of raspberry grown during the 1930 season in inoculated sandy-loam soil indicate that Ranere displays some resistance to the disease (table 6). In this experiment the root systems of the Cuthbert plants used were

but one third as extensive as those of the Ranere. Had the plants been equal in size the difference between the two varieties in all probability would have been more sharply defined. The Latham variety was not intentionally used in these tests. A few plants developed from roots left in these plots from an earlier planting.

TABLE 6.—*Development of crown gall on 5 varieties of raspberry grown in inoculated soil, 1930*

Variety	Total plants grown	Average galls per plant	Plants infected	Plants visibly injured by root-feeding arthropods
	Number	*Number*	*Percent*	*Percent*
Cuthbert	90	7.0	98	62
Cumberland	52	6.6	94	85
Latham	8	6.6	88	75
Plum Farmer	48	4.9	75	77
Ranere	84	3.2	72	39

Injuries are inevitably caused to the root systems of raspberry plants in storage and in transit. To determine whether injuries to the roots caused prior to planting are subject to infection, 12 plants received from a nursery were injured just before they were planted in the inoculated soil plots described under the heading Seasonal development (p. 779). The injuries were marked by loosely encircling the stem or root at the point of injury with an aluminum wire band. Inspection 14 weeks after planting showed that 19 of the 48 injuries (40 percent) so treated had become infected. Of the 71 injuries made on plants set out in plots of soil that had been steamed none became infected.

INFLUENCE OF MECHANICAL INJURY

Experiments were made to determine whether crown-gall bacteria enter the red raspberry only through injuries. Under controlled conditions in the greenhouse experiments, crown-gall infection did not occur in the absence of either of two factors; namely, (1) injuries to the underground parts of the plant and (2) crown-gall bacteria in the soil in which the plants were grown. Of plants grown in inoculated soil and not guarded against injuries to their underground parts, 80 percent developed the disease. The average number of infections on these diseased plants under controlled conditions was about equal to that found for diseased plants grown in inoculated soil in the field.

These experiments were carried out in the greenhouse on a limited number of plants known to be practically free from crown-gall infections. The plants were disinfected by immersion in silver nitrate solution (1:1,000) for 20 minutes and then planted in 10- or 12-inch pots or in root-observation boxes in soil that had previously been steamed. Prior to the time of the experiment the plants had been grown for 1 or 2 years in the steamed-soil plots previously described. During this period the incipient stages of infection developed that were carried on the plants from the nursery. By discarding the infected plants and selecting only those that had grown at a distance of 3 feet or more from them, plants were obtained having a minimum of incipient macroscopically nonvisible infections. The extent of this minimum quantity of infection was ascertained at the close of each

experiment by recording the number of infections found on plants that had not been exposed to crown-gall bacteria during the course of the experiment. Tables 7 and 8 show that no infections were present on the plants selected 1 year after planting in steamed soil, but that a small number of such infections were present on plants removed at the end of 2 years from the steamed-soil beds.

The plants were guarded against the effects of mechanical injury of two types: (1) Injury received when the plants were transplanted at the beginning of the experiments and (2) injury caused by root-feeding arthropods. The injury received in transplanting was practically eliminated as a factor in infection by holding the steam-sterilized soil in which these plants were grown free from crown-gall bacteria for 3 or 4 months. A few injuries received in transplanting became infected when the soil was inoculated 3.5 months after the time of transplanting. Practically, however, this procedure furnished an adequate control against this kind of infection. Injury caused by root-feeding arthropods was guarded against in the 1927–29 experiments by setting the pots or boxes on planks held 3 feet above the greenhouse floor by wooden supports 4 inches square. The boxes or pots were held three fourths of an inch above the surface of the planks by wooden supports 1 by 2 inches. In the experiments conducted in the 1930 season, protection against arthropods was attained by steam sterilization of the soil used, disinfection of plants and containers, and enclosing the plants and their containers within cheese-cloth cages. The plants that were not guarded against root-feeding arthropods were held on the ground (covered by a dense growth of weeds and sod) outside the greenhouse or on the soil floor within the greenhouse.

In the experimental procedure the three main groups of plants were subjected to the variations in conditions shown in tables 7 and 8.

TABLE 7.—*Influence of (1) injuries caused chiefly by root-feeding arthropods and (2) the presence of crown-gall bacteria in the soil on crown-gall infection of red raspberry, 1927–29*

| Total plants | Injuries caused by— | | Crown-gall bacteria in soil | Infections per plant (average number) |
	Transplanting	Root-feeding arthropods		
36	Present	Present	Present	6. 1
18	do	do	None	. 0
25	None	None	Present	. 2

TABLE 8.—*Influence of injuries caused chiefly by root-feeding arthropods on development of crown gall on red raspberry plants grown in inoculated soil in 1930*

| Plants grown | Conditions of growth | | Injuries caused by | | Crown-gall bacteria | Galls per plant (average number) |
	Place	Time of transplanting	Transplanting	Root-feeding arthropods		
274	Field	Early [a]	Present	Present	Present	4. 8
47	do	Late [b]	do	do	do	2. 5
27	Greenhouse	do	do	do	do	2. 4
39	do	do	do	do	None	. 3
91	do	do	None	None	Present	. 2

[a] For a more detailed record of these plants see table 6.
[b] Latham plants were used in all the greenhouse series and in the group of plants transplanted late in the field.

The results of the first series of experiments made in the seasons of 1927 to 1929 are summarized in table 7. Infection was present only on those plants subjected to both injury and the crown-gall bacterium. Four infections occurred on plants subjected to inoculum and guarded against mechanical injury caused by arthropods and against the effect of injury received in transplanting. These galls were 3 mm or less in diameter when the plants were harvested, and occurred at points of contact of root surface and the glass sides of the root-observation boxes used in the experiment. It is probable that these infections took place through minute abrasions of the root surface caused by sand grains. When pressure was applied to the doors covering the glass sides of the boxes, sand grains might have been forced into the roots at these points of contact. Infection occurred in 90 percent of the injuries caused for control purposes on the underground parts of these plants just prior to the inoculation of the soil. Of 201 injuries caused to these plants at various intervals up to 21 days prior to the application of inoculum to the soil, 134 were galled at the close of the growing season. From the ready response of the plants to inoculation and of the injuries caused on these plants to infection (table 5) it is apparent that the lack of infection at other points than those intentionally injured or inoculated could not be attributed to the late application of inoculum to the soil in which the plants were grown. The results of these experiments indicate that there are no natural openings through which infection takes place in early autumn on the underground parts of the red raspberry.

These experiments were repeated on a larger number of plants in 1930. The methods used and the results obtained were essentially the same (table 8). The first set of plants used in this experiment was stunted by lack of adequate drainage in the new type of root-observation box used, and a second series was planted in the same boxes in early June after this defect in construction had been corrected. The smaller amount of infection on the plants in this series subjected to both injury and crown-gall bacteria is believed to have been due to the lower vitality of the plants, which resulted from late transplanting. Under field conditions in inoculated soil the amount of infection that occurred on a number of the same lot of plants was practically equal to that found on the plants exposed to inoculum and arthropods in the controlled series as previously described.

INFLUENCE OF ROOT-FEEDING ARTHROPODS

Many observations suggest that root-feeding arthropods cause most of the injury to the underground parts of the raspberry through which crown-gall infection occurs. Injuries caused by such arthropods have been found in every planting of raspberries studied by the writer at Madison, Wis. In some plantings 85 percent of the plants were visibly injured in this way. Crown-gall infection frequently occurred at such points of injury to the underground parts of plants grown in inoculated soil, but most of the injuries did not become infected. The injury appeared to be caused by a number of root-feeding arthropod forms.

The injury found on the plants was of several types, ranging from very minute abrasions of the surface of delicate rootlets to severe pruning of lateral roots at the crown of the plant and decortication of

the underground stem and larger roots. The more insignificant types of injury appeared on tiny roots and could be detected with the naked eye only by the most minute and careful examination (fig. 3, *A*). On the smaller roots (2 mm or less in diameter) cortical incisions were frequently abundant. These occurred singly (fig. 3, *L*) or in series close together and appeared as dark brown parallel lines at right angles to the longitudinal axis of the root (fig. 3, *I, J, N*). Occasionally the smaller roots were decorticated for short distances (fig. 3, *K*) or had small shallow cavities gouged out of their surfaces (fig. 3, *E, F, G, M, O*). These lesser injuries were characteristic of those found on plants grown on the low reclaimed soil of the marsh described in an earlier section of this paper. The most conspicuous type of injury was found on plants grown on the well-drained sandy-loam plots and consisted of large shallow cavities gouged out of the larger roots and the underground portions of the stems (fig. 4, *A* to *E*). Besides such injury, many branch roots, often 50 percent or more, were completely severed from the plant (fig. 4, *F*). This type of injury was not observed during the seasons of 1928 and 1929. The majority of the plants grown on the sandy-loam soil in 1930 were injured in this manner (table 6).

Crown-gall infection developed on a large percentage of injuries to the underground parts of red raspberry plants grown in inoculated soil (figs. 3 and 4). Usually after several months of development the galls had completely obscured the injury through which the infection took place. Gall development obscured within a few weeks the smaller injuries through which infection occurred (fig. 3, *A* to *H, N* to *S*). In the seasonal-development study discussed later there was no indication of the manner in which infection occurred in over 95 percent of the galls. The injuries through which infection entered the plants grown in the reclaimed marsh soil were of an inconspicuous type (fig.3).

In two types of experiment, involving the exclusion and inclusion of arthropods, respectively, it has been shown that root-feeding arthropods cause the injuries through which crown-gall infection occurs on the raspberry. In the exclusion experiments, groups of Latham red raspberry plants were grown in inoculated soil from some of which arthropods were excluded and from some of which they were not excluded. The methods have been already described. When no precautions were taken to exclude root-feeding arthropods from these plants, infection occurred as abundantly as under the usual field conditions. When the plants were protected from such arthropods in the soil in which they were grown no infection was observed (tables 7 and 8). The field observations and experimental results indicate that under ordinary conditions root-feeding arthropods are the chief factor in creating the injuries through which crown-gall infections occur. In these experiments no attempt was made to ascertain what arthropod species caused the injuries in question.

The arthropod-inclusion experiments consisted in confining root-feeding species (1) to limited areas of root surface and (2) to the soil in the container in which the plants were grown. The arthropod forms used were click-beetle larvae (Elateridae), millepedes (Diplopoda), and white grubs (*Phyllophaga* sp.). In the first type of experiment the larvae were confined to short uninjured lengths of lateral roots from 1 to 3 mm in diameter, by enclosing a length of 2 to
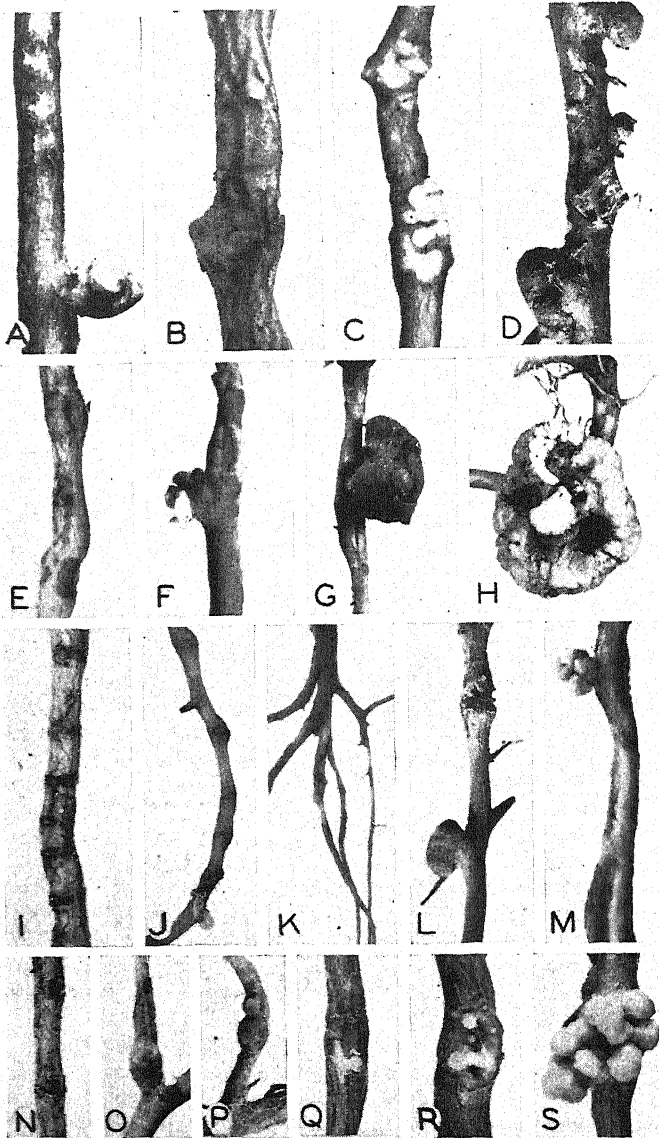
FIGURE 3.—Representative injuries caused by root-feeding arthropods on roots of Latham red raspberry plants grown in the field in soil inoculated with *Phytomonas tumefaciens*. Crown-gall infection was found commonly on these plants in association with injuries caused by root-feeding arthropods. Crown gall has developed from infection through a number of injuries shown. Injuries: *A*, Minute abrasions of the root cortex (upper half of figure) at top of stem, barely visible to the naked eye; *B, C, E, F, G, M, O, P*, small shallow cavities chewed from the roots; *I, J, N*, multiple incisions in the roots; *K*, decorticated small lateral roots. Infections: *B, K, Q*, Crown-gall overgrowth just appearing from infection through injuries; *C, G, O, R*, crown-gall overgrowth is rapidly obscuring the injuries through which infection occurred; *D, H, L, S*, lower *A, F, J*, upper *M*, crown-gall overgrowth has obscured the injury through which infection occurred; *Q, R, S*, stages of crown-gall formation after infection through injuries caused by a scalpel; *H*, circular depressions have resulted from the feeding of grubs on a crown gall. Scale of enlargement: *K*, × 1; *H, Q, R, S*, × 2; *C, D*, × 3; *G, I, J, O, P*, × 4; *B, E, F, M, N*, × 5; *A, L*, × 7.

4 inches of one or several such roots in a wire screen.   The wire screen
used was of brass (100 meshes per square inch), and was sewed with
brass wire in the form of a tight sack, 4 by 3 by 1.5 inches, about the
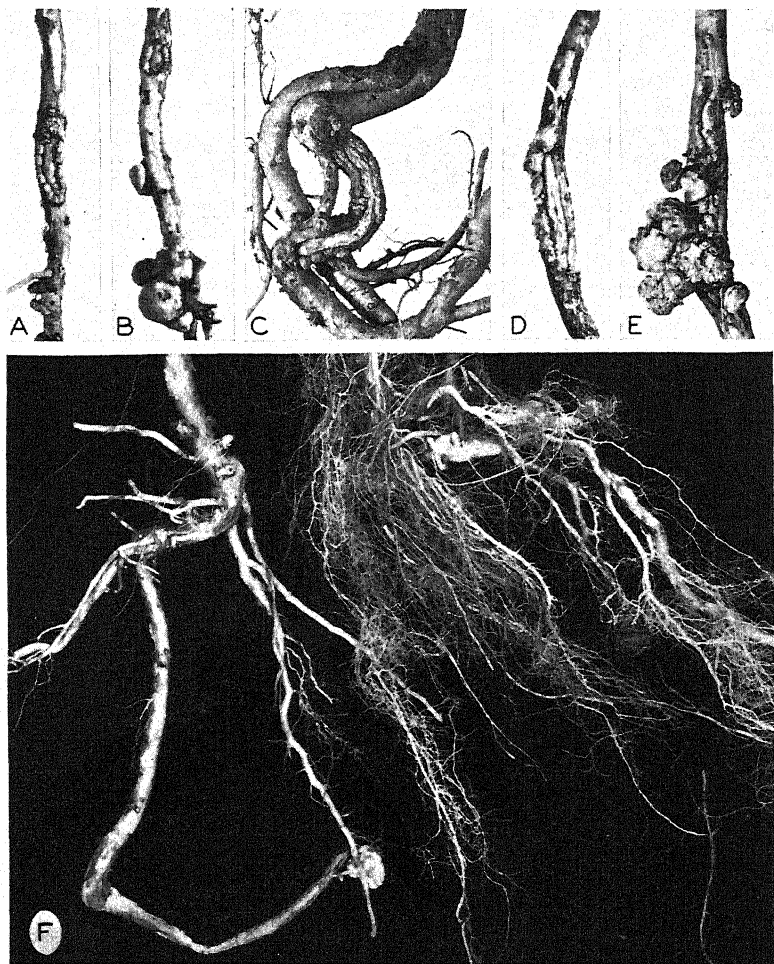
FIGURE 4.—Typical white-grub injury on the roots of raspberry plants. Crown-gall infections were usually
associated with this type of injury in 1930 at Madison, Wis., on all raspberry plants grown in the field in
soil inoculated with *Phytomonas tumefaciens*.   Injuries: Shallow to deep cavities were gouged by white-
grubs, *Phyllophaga* sp., from the larger roots, and the underground portions of the stem of both red (*A,
B, F*) and black (*C, D, E*) raspberry varieties.   Frequently 50 percent or more (*F*, left) of the lateral
roots were eaten from plants in field plots.   *A, B,* Injuries caused by grubs on the roots of Latham red
raspberry plants grown in a container of inoculated soil in which white grubs had been placed.   Infections;
B, Crown-gall development at top not perceptible from infections through injury; *A, C,* Barely percep-
tible at the injury; *D,* rapidly covering the injury; and *B, E,* obscuring the injury.   *F,* Representative
root system of a series of plants grown in the greenhouse in pots of inoculated soil and subjected to the
following conditions: White grubs were placed in the soil (left) and root-feeding arthropods were excluded
from the soil (right).   Crown-gall infection has occurred through several of the injuries caused by the
grubs.   No injuries and no infection occurred on plants from which arthropods were excluded.   Scale
of enlargement: *A, B, D,* and *E,* × 2; *C,* natural size; *F,* ½ natural size.

roots.   The smaller spaces around the points where the roots pro-
truded from the sack were stuffed with glass wool.   Two wireworms
were put into each of two such wire-screen sacks, which were then

filled with soil and sewed shut. One millepede was put into each of two other screen cages. One white grub was put into each of four similar root enclosures. The soil in the sacks was then inoculated by applying to the soil of the root-observation boxes used a suspension of crown-gall bacteria. Six weeks later the screen cages were removed and observations were made. The roots in one of the wireworm cages were not noticeably injured. In the other, one side of the root enclosed was decorticated for a length of 2 inches and crown-gall infection had developed at two points along this area of injury. Since the injury extended past the end of the screen cage, it is possible that it may have been made in fastening the cage about the root. The millepede in one cage had decorticated the root in two places, no infection had occurred, and the root had died. The white grubs had eaten all the roots enclosed within each of the four screen cages in which they had been confined, leaving not a trace. Of the four, two escaped into the soil in the free space of the root-observation box. Examination of the roots in the box revealed injuries identical in type with those found on the majority of plants grown in the field in 1930 (fig. 4, *A*, *B*). Crown-gall infection had occurred through two of seven such injuries. All the arthropods employed in the foregoing studies, except one millepede, were recovered alive at the close of the study.

A further experiment was made in which white grubs were placed at large in pots in which disease-free raspberry plants were growing. The soil was then inoculated, as was the soil in which a control group of plants were growing. The control group was protected from all arthropod forms. In each group there were ten 6-inch pots, each containing two plants. Six weeks after two large white grubs had been placed in each of the pots of the first group and the soil of both groups had been inoculated with a suspension of crown-gall bacteria, all plants were washed free of soil and examined. Of the 20 plants grown in pots in which grubs had been placed, all were conspicuously injured. Large shallow cavities had been gouged from the underground part of the stem and the larger roots. Frequently the roots had been either chewed completely off or about half eaten away for a length of 1 to several inches. The lateral roots of smaller diameter had been largely eaten away (fig. 4, *F*). Of these 20 plants 7 had developed crown-gall infection at the points of white-grub injury, with an average of 3.6 galls per plant; 2 of the remaining 13 had died. No injuries were found on the underground parts of the plants in the parallel group that had been protected from all arthropod forms, and no crown gall developed on these, though both groups had been exposed alike to inoculum. The injury caused by these grubs was identical with that found on plants grown in the inoculated field plots in which white grubs were found in abundance (fig. 4, *B* to *E*; table 6). From these experiments and observations it is concluded that in 1930 white grubs caused most of the injury through which crown-gall infection developed on raspberry plants grown in inoculated soil at Madison, Wis.

INCUBATION PERIODS

During the studies, already described, on the length of time wounds remain open to infection, variation in the incubation period of crown-gall infections on the underground parts of red-raspberry plants was

observed to occur. This variation appeared to be correlated with
(1) the general condition of the host plant in response to environ-
mental factors, particularly those relating to the season, and (2) local
variation within individual plants due to differences in age and vigor
of roots. It was observed that overgrowths of appreciable size ap-
peared within 11 days after inoculations made on August 23 (table 5),
whereas inoculations made on October 4 did not induce appreciable
overgrowths until 3 weeks later. From the data recorded in table 5
it is clear that many inoculations which had not induced macroscopi-
cally visible overgrowths 3 to 4 weeks after the application of inoculum
developed such overgrowths later. In these studies the incubation
period of crown-gall infections on the roots of the red raspberry was
observed to range from 11 days to more than 4 weeks.

A knowledge of the prolonged incubation period, which appears to
lengthen as the plants approach the dormant condition, and of the
relatively long period in which injuries to the underground parts of
the red raspberry may remain subject to infection by crown-gall
bacteria, is important in understanding the incipient stages of infec-
tion by which the disease is disseminated to new areas.

SEASONAL DEVELOPMENT

Little has been reported heretofore concerning the time at which
crown-gall infection occurs, and the course of the development of this
disease on the red raspberry has remained obscure. From seasonal
development studies reported herein it was found that crown-gall
infection occurred (1) at random on all underground parts of the
plants and (2) at a practically uniform rate throughout the course of
the two seasons studied.

For a period of 2 years a study was made of the seasonal develop-
ment of crown gall in a planting of Latham red raspberries. All the
plants studied were without macroscopically visible infections when
they were planted in a field plot of inoculated Clyde silt-loam soil.
The plot was inoculated 1 day before planting by mixing into the
upper 9 inches of soil a suspension of crown-gall bacteria applied at
the rate of 1 pint per square foot of soil. The suspension used was
made and applied as described earlier for the overwintering studies.
From time to time during the season in which these plants were set out
and during the following season a number of plants were dug at
random from the plot and their condition with regard to the disease
was recorded. Each plant was washed free of soil particles in a
stream of water, and all its parts were then examined minutely for
macroscopically visible infections. In this way all visible galls on the
plant were recorded with notations as to their size and position.
From 40 to 75 plants were examined and discarded at each observa-
tion. The data obtained from this study are presented in condensed
form in tables 9, 10, and 11, and in figure 5.
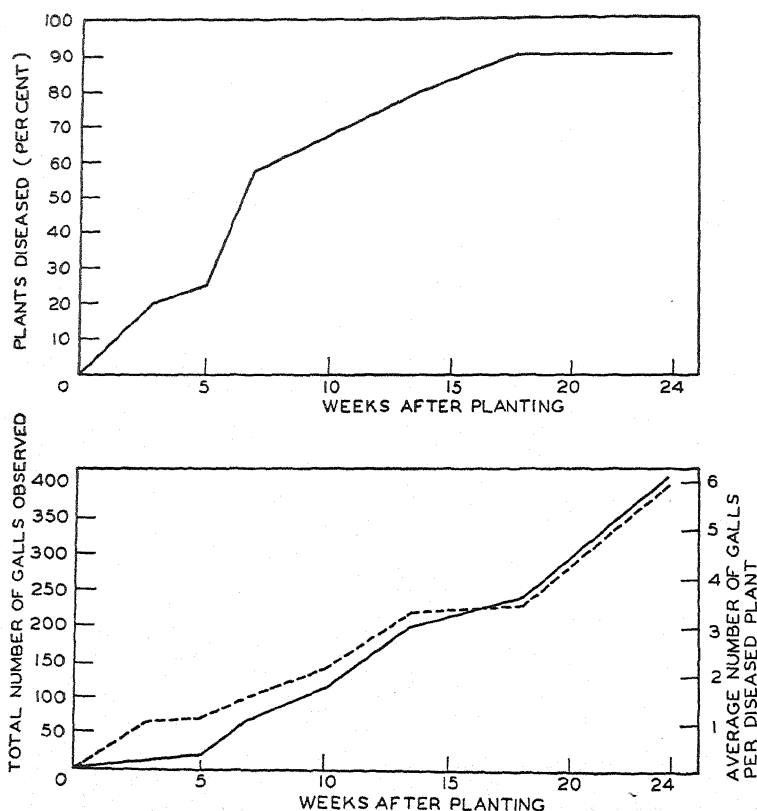
FIGURE 5.—Seasonal development of crown gall on underground parts of red raspberry plants grown in the field in soil inoculated with the crown-gall organism. Broken line in lower graph indicates average number of galls per diseased plant.

TABLE 9.—*Seasonal development of crown gall on underground parts of red raspberry plants grown in inoculated soil, 1928–29*

Date	Length of time after planting	Plants examined		Galls present					
		Total	Infected	Total	Average per infected plant	Distribution according to indicated diameter			
						0.5 to 2 mm	2.1 to 5 mm	5.1 to 10 mm	Larger than 10 mm
1928	*Weeks*	*Number*	*Percent*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
June 6_____	3	40	20	8	1.0	3	2	3	0
June 23____	5	40	25	11	1.1	8	1	2	0
July 6_____	7	75	56	68	1.6	49	8	7	6
July 27____	10	40	68	61	2.2	28	15	11	7
Aug. 17____	13	75	80	218	3.6	54	67	68	29
Sept. 17___	18	75	91	247	3.6	36	83	61	67
Oct. 28____	24	75	92	402	5.8	66	99	105	132
1929									
June 1_____	55	50	90	285	6.3	34	59	133	43
Aug. 1_____	64	50	94	a 271	5.7	3	54	110	34
Nov. 1_____	78	50	96	302	6.3	12	24	180	86

a The total number of galls here includes 70 which had disintegrated to the stage where they could be detected only by scars left on the roots.

TABLE 10.—*Size and location of crown galls observed during the first season on underground parts of red raspberry plants grown in inoculated soil, 1928* [a]

Length of time after planting (weeks)	Location and size of crown galls											
	Galls of indicated diameter on stem				Galls of indicated diameter on branch roots larger than 2 mm				Galls of indicated diameter on branch roots less than 2 mm			
	0.5 to 2 mm	2.1 to 5 mm	5.1 to 10 mm	Greater than 10 mm	0.5 to 2 mm	2.1 to 5 mm	5.1 to 10 mm	Greater than 10 mm	0.5 to 2 mm	2.1 to 5 mm	5.1 to 10 mm	Greater than 10 mm
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
3	2	2	2	0	1	0	1	0	0	0	0	0
5	2	1	2	0	2	C	0	0	4	0	0	0
7	6	2	3	3	2	4	3	2	41	2	1	0
10	C	1	5	4	7	11	4	3	21	3	2	0
13	2	10	17	10	21	20	25	15	31	37	26	4
18	0	11	14	19	6	24	25	43	30	48	22	5
24	4	12	10	39	16	37	64	74	46	50	31	19

[a] For dates of observation, number of plants examined, number of infected plants, and total number of galls observed, see table 9.

TABLE 11.—*Size and condition of crown galls observed during the second season on underground parts of red raspberry plants grown in inoculated soil, 1929* [a]

Date of observation	Condition of galls observed	Total galls observed	Average galls per plant	Distribution of galls according to diameter			
				0.5 to 2 mm	2.1 to 5 mm	5.1 to 10 mm	Larger than 10 mm
		Number	*Number*	*Number*	*Number*	*Number*	*Number*
June 1	Living	132	2.9	28	34	53	17
	Dead	153	3.4	6	25	78	44
Aug. 1	Living	123	2.6	2	41	57	23
	Dead	[b] 148	3.1	1	13	53	11
Nov. 1	Living	170	3.5	12	19	107	32
	Dead	132	2.8	0	5	73	54

[a] The number of plants examined, number of diseased plants, and the average number of infections per plant are given in table 9.
[b] 70 galls which had disintegrated to the stage where their presence could be distinguished only by the scars that their decay had left on the roots could not be recorded as to size.

As an ecological check, two smaller plots of Miami sandy loam were inoculated and planted at the same time as the silt-loam plot described above. These smaller plots were situated on a gentle slope about 1 mile distant from the drained low marshland on which the silt-loam plot was located. The same soil treatments and the same plant materials were used in both experiments. The plants in the smaller plots were not examined until the close of the second season, however, at which time all were harvested and a detailed record was made of their condition with reference to the disease. The comparative record of the development of crown gall on plants grown in these inoculated sandy-loam and silt-loam soils is shown in table 12.

TABLE 12.—*Comparison of crown galls found on red raspberry plants grown in sandy-loam and in clay-loam inoculated soils* [a]

Age of plants and soil type	Plot no.	Total plants	In-fected plants	Total galls	Aver-age galls per in-fected plant	Distribution according to diameter of crown galls observed							
						Living				Dead			
						0.5 to 2 mm	2.1 to 5 mm	5.1 to 10mm	Larger than 10 mm	0.5 to 2 mm	2.1 to 5 mm	5.1 to 10mm	Larger than 10 mm
		No.	*Pct.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
2-year plants:													
Sandy loam	22	37	95	285	8.1	32	28	94	31	0	2	49	49
Do	21	34	94	[b] 283	8.8	51	53	118	61				
Clay loam	M	50	96	302	6.3	12	19	107	32	0	5	73	54
1-year plants:													
Sandy loam	22	28	57	35	2.2	14	6	9	6	0	0	0	0
Do	21	36	67	[c] 131	5.4								
Clay loam	M	94	17	22	1.4	3	2	13	4	0	0	0	0

[a] Data were recorded at time of harvesting the plants at close of second growing season subsequent to planting in inoculated soil.
[b] No record was made of the condition of these galls.
[c] No record was made of the condition or size of these galls.

A further check to ascertain the number of undeveloped infections that these plants carried when shipped from commercial nursery storage was made in parallel plantings on sandy-loam plots that had been steamed. These plots were at a slightly higher level and were adjacent to the inoculated sandy-loam plots described above. The development of the disease on the plants in these steamed plots has been discussed already. All plots were planted from a shipment of Latham red raspberry plants received from a nursery in May 1928.

The following is a summary and brief discussion of the results of the study of the seasonal development of crown gall on red raspberry plants grown in inoculated soils.

Infection occurred at all times and at a practically uniform rate throughout the entire growing season.

The number of plants showing crown-gall infection increased rapidly from 20 percent in early June of the first season (an increase at that time of 5 percent over the controls planted in the steamed beds) to about 90 percent by the middle of September. The number of diseased plants then gradually increased to 96 percent by the close of the second season (table 9, fig. 5).

The number of infections per plant increased steadily and at a practically uniform rate throughout the growing season. This increase appears as a relatively uniform progression when the number of infections is plotted against time (fig. 5). The average number of infections found per diseased plant in June was 1.0 as contrasted with 5.8 at the close of the season. In June of the second season a maximum of 6.3 infections per diseased plant was reached. Although new infections continually appeared during the second season (table 11) the number of galls found per diseased plant remained about constant. This may have been due in part to decomposition of galls formed as a result of infections that occurred during the first season.

Infection appeared to occur at random on all underground parts (fig. 6). It did not seem to be confined to any particular developmental phase or portion of the plant. On the underground section of the stem, on the branch roots of large or small diameter, infection
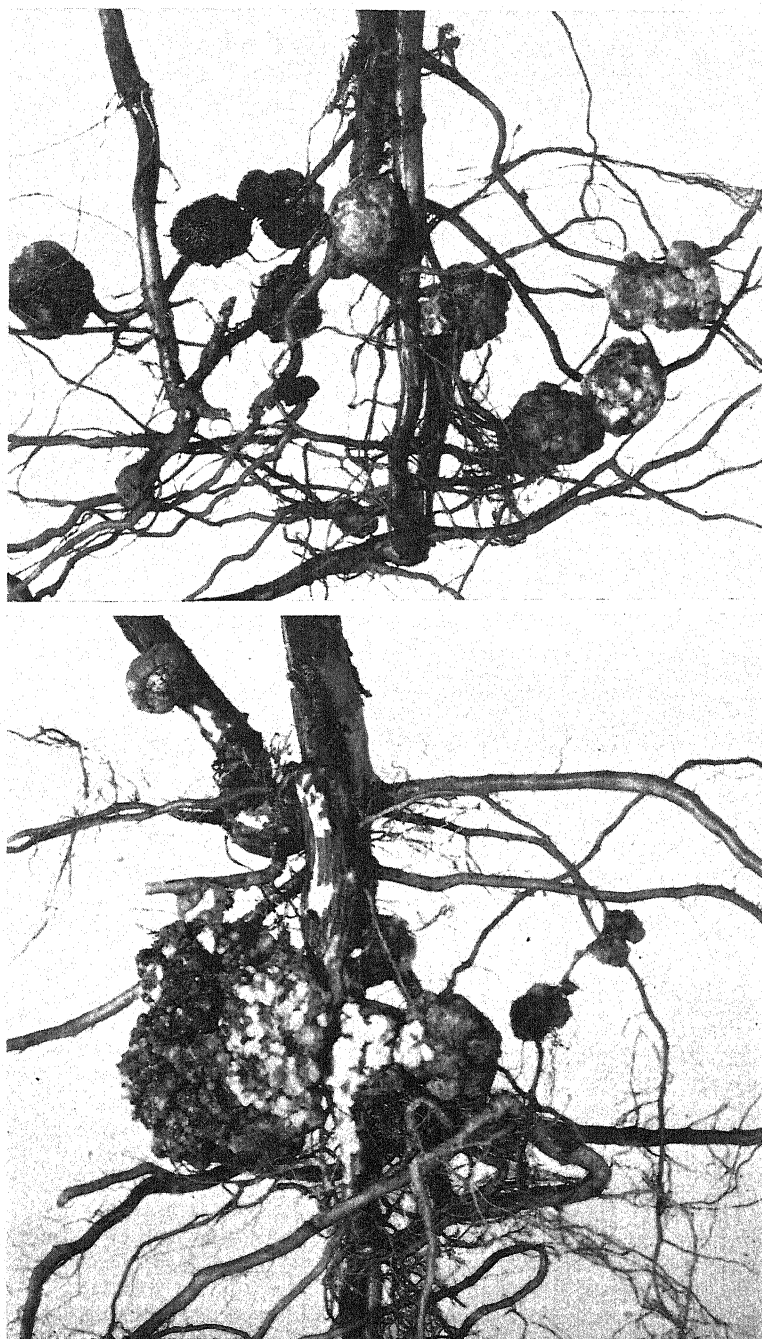
FIGURE 6.—Typical crown gall on the roots of Latham red raspberry plants grown in the field in soil inoculated with *Phytomonas tumefaciens*. This type of crown gall occurred on the roots of over 95 percent of the plants grown under these conditions. Aerial portions of the plants were affected very rarely—less than 0.2 percent of all plants grown.

occurred alike and continuously throughout the season (table 10). As discussed in an earlier section, infection appeared with greatest frequency on those underground portions of the plant within 4 inches of the surface of the soil.

Infection was repeatedly observed to have taken place through injuries caused by root-feeding arthropods.

The disease developed more extensively on plants grown on the higher well-drained sandy loam (table 12) than on plants grown on the reclaimed silt loam of the marsh. At the time of harvesting, the disease seemed from general observation to be more severe on the plants grown in the sandy loam. That the disease did develop more extensively on the plants in these plots is apparent from the comparative record (table 12), from which it is shown that:

The number of first-year plants diseased was 57 to 67 percent, respectively, for the sandy-loam plots and 17 for the silt-loam plot; the average number of infections per diseased first-year plant was 2.2 and 5.4, respectively, for the sandy-loam plots, and 1.4 for the silt-loam plot; the average number of infections per diseased 2-year plant was 8.1 and 8.8, respectively, for the sandy-loam plots and 6.3 for the silt-loam plot; toward the end of the second season the rate at which infection was occurring in the sandy-loam plots was about three times that in the silt-loam plot. This rate is a function of the number of 0.5 to 2.0 mm galls found on the plants at any observation.

SUMMARY

Crown-gall bacteria are given off continuously from the surface of living crown galls under suitable moisture conditions.

Crown-gall bacteria were found to overwinter in the soil of fallow fields at Madison, Wis., and to exist in a pathogenic state for 14 months in unsterilized soil held free from the growth of seed plants under nursery storage conditions.

Crown gall is carried from the nursery to new areas as incipient undeveloped infections on the raspberry that cannot be detected by inspection.

Crown-gall infection was found to occur at a practically uniform rate throughout the course of the growing season on red raspberry plants grown in inoculated soil.

Crown-gall infection occurs on the raspberry only through injuries. No infection was found on plants which were exposed to inoculum but the underground parts of which were protected from injury.

Injuries on the underground parts of the red raspberry remain subject to crown-gall infection for a relatively long period. A large percentage of the injuries studied became infected when inoculum was applied to their surfaces 3 weeks after the injury was made. A small percentage of those studied became infected when inoculum was applied to them 7 weeks after the injury was made.

The incubation period of crown-gall infection on the underground parts of the red raspberry was found to vary from 11 to more than 28 days as a result of environmental conditions, chiefly seasonal.

In these investigations root-feeding arthropods caused most of the injuries through which crown-gall bacteria entered. The injuries found on the underground parts of the plants probably were caused by a number of species of arthropods. White grubs caused most of the injuries through which infection occurred in 1930.

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CHROMOSOMES IN HYBRIDS BETWEEN EUCHLAENA PERENNIS AND ZEA MAYS[1]

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INTRODUCTION

Hybrids between maize (*Zea mays* L.) and perennial teosinte (*Euchlaena perennis* Hitchc.) offer a favorable opportunity to follow the behavior of triplicate sets of chromosomes in a triploid hybrid. Maize, the diploid parent, has been carefully analyzed both genetically and cytologically. Perennial teosinte seems to be unquestionably a true tetraploid. Its tetraploid character is evident from (1) the prevalence of tetravalent chromosomes at diakinesis observed by Randolph[3] and by the writer; (2) the X-ray experiments of Randolph (*17*),[4] who obtained from the 20-chromosome annual *Euchlaena* a 40-chromosome perennial plant very like *E. perennis*; and (3) genetic studies which show that F_1 teosinte-maize hybrids contain three sets of homologous chromosomes. Although extensive genetic studies were carried on in conjunction with the cytological studies, the present paper treats only of the chromosomes as found in F_1 and later hybrid generations.

The first plants of perennial teosinte were found in Mexico by Hitchcock in 1910 and were introduced into this country by Collins (*4*) in 1921. The first hybrid with maize was made early in 1922 by C. G. Marshall. The breeding has been done largely at the United States San Diego Acclimatization Garden at Torrey Pines, Calif., where the more promising perennial hybrid forms are maintained in permanent plantings. The methods of crossing have been similar to those used in corn-hybridizing studies, but to bring plants with very different flowering dates into flower at the same time, plantings were made at different seasons and late-flowering plants were frequently given an artificial short day.

The cytological material, which had been collected just before the tassel emerged from the leaves, was put in Carnoy's killing fluid for half an hour; this fluid was then poured off and the material was covered with absolute alcohol. Material preserved in this way is generally satisfactory for the determination of chromosome numbers by the use of the acetocarmine smear method.

CHROMOSOME BEHAVIOR IN POLLEN MOTHER CELLS

The hybrids between maize and teosinte that were used in this study were F_1, F_2, back crosses, and more complicated crosses. The determination of the chromosome numbers of individual plants was

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most readily made at the first-division anaphase. Figure 1, A–D, shows this phase in an F_1, a back cross, a back cross again back-crossed to maize, and a self of a back cross, respectively.
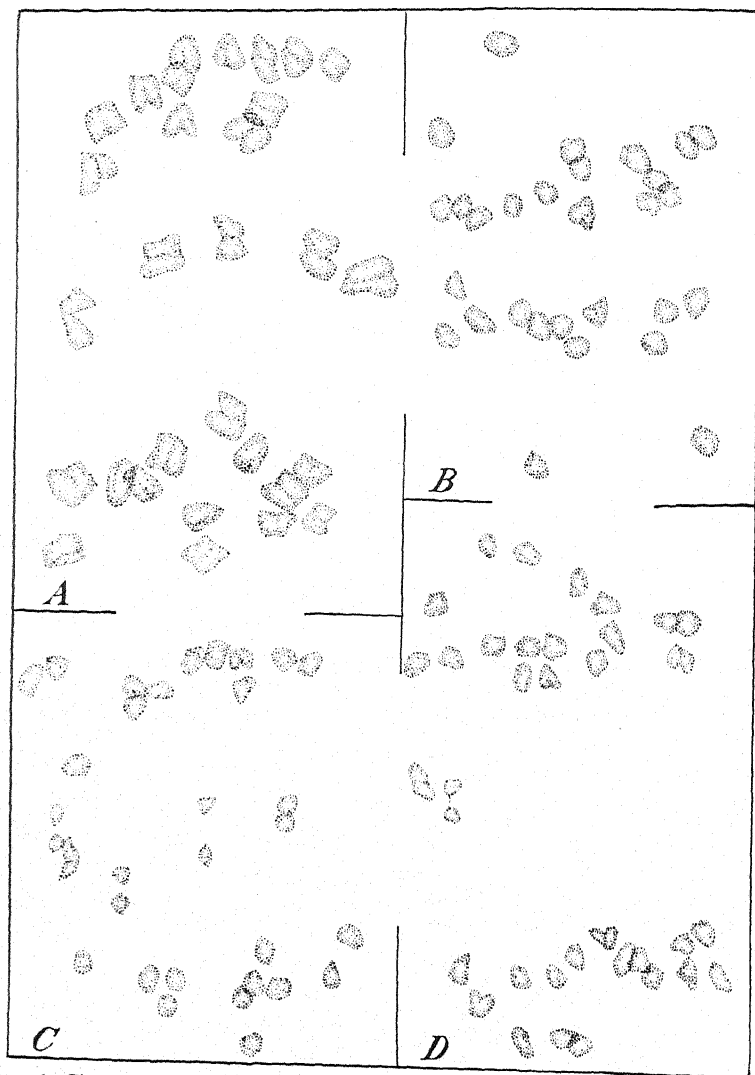
FIGURE 1.—First-division anaphases in teosinte-maize hybrids: A, 30 chromosomes of an F_1 plant; B, 29 chromosomes of a back cross; C, 28 chromosomes of a back cross crossed with maize; D, 35 chromosomes of a selfed back cross. $A \times 1,900$; B–$D \times 1,200$.

At this phase all bivalent chromosomes have divided and the univalents appear almost identical with the halves of the bivalents, so that a count of all chromosomes gives at once the $2n$ number of the plant under inspection. If the figure happens to be in late anaphase, a few of the univalents may be in the region of the plate in the process

of division. Even at this stage the character of the dividing univalents is such that they are not mistaken for bivalent chromosomes.

In 1924 the writer (*13*) reported the number and some of the characteristic features of the chromosomes in the developing pollen mother cells of F_1 plants of crosses between *Euchlaena perennis* and *Zea mays*. Since that time additional material has been available, and it has been found that with one exception, referred to later, all plants have 30 chromosomes, the sum of the haploid chromosome numbers of the two parents.

The behavior of the chromosomes in these hybrids during meioses of the pollen mother cells has been found to be that already described (*13*), except in a few minor details. A closer examination of the disposition of the univalent chromosomes, which are usually scattered on the spindle at the time the bivalent and trivalent chromosomes form the first-division metaphase plate, shows that many of them move undivided to the poles with the halves of the divided chromosomes. However, one or more of the univalents frequently will be found at the plate region when the divided chromosomes are in the anaphase, and at late anaphase these univalent chromosomes will divide. Except for the occasional undivided univalents or halves of univalents that are too far afield, the two daughter nuclei include the divided halves of the bivalent chromosomes and varying numbers of divided and undivided univalent chromosomes. Those chromosomes that fail to be included in the daughter nuclei are left in the cytoplasm to form micronuclei or to degenerate.

The second division is much more regular than the first. Only the halves of univalents present cause any appreciable abnormalities. Their position on the metaphase spindle is indefinite and their distribution is at random to the two poles.

The chromosome behavior during meiosis of the pollen mother cells of later hybrid generations is in most respects similar to that observed in F_1 hybrids. The chromosome numbers in F_2, in back crosses, and in others of the more complicated hybrids are likely to vary for each individual. The chromosome complement of such plants will be made up of chromosomes derived from the two parents. The number from either parent is unpredictable, and unfortunately cytological study has not yet developed any method of differentiating the chromosomes of maize from those of teosinte.

Camera-lucida drawings were made as a record of the chromosomes of each plant, since the temporary slides could not be preserved for any appreciable time. The drawings of first-division anaphases gave an opportunity to study the distribution of chromosomes to the two daughter nuclei resulting from the first meiotic division.

The distribution of the chromosomes in an F_1 hybrid seems generally to show that of the 3 sets of 10 chromosomes present 2 sets pair regularly and 10 chromosomes go to each pole, whereas the chromosomes of the third set are distributed at random to the two daughter nuclei. Figure 2, *A*, shows 10 bivalents, 1 pair of loosely associated chromosomes, and 8 univalents. In plants of later hybrid generations, however, the number of regularly pairing chromosomes may be more than 10, since a plant may have 4 or more homologous chromosomes instead of just 3 as in F_1 hybrids. Figure 2, *B* and *C*, shows cells having 22 chromosomes. In *B* all chromosomes are paired, and in *C* there are 10 pairs and 2 univalents, suggesting that in the former

there are 4 homologous chromosomes present, whereas in the latter the 2 unpaired chromosomes are not homologous.

The genetic data have shown that in F_1 plants and in back crosses on maize autosyndesis is the rule and allosyndesis the exception, so that in F_1 hybrids the unpaired chromosomes are from maize, whereas in plants from back crosses on maize the unpaired chromosomes are from teosinte.

Table 1 gives a comparison between the numbers of chromosomes that go to make up the daughter nuclei resulting from the first meiotic division of 30-chromosome hybrids and the numbers calculated when 10 paired chromosomes go at random to join the 10 divided bivalents at the two poles, i.e., the binomial expansion of $(a+b)^{10}$. Although the data of this table are made up from drawings of plants with different percentages of maize and teosinte chromosomes, it indicates that the number of chromosomes between 10 and 20 that enter a gamete depends upon chance. The binomial expansion does not represent the chance distribution of extra chromo-
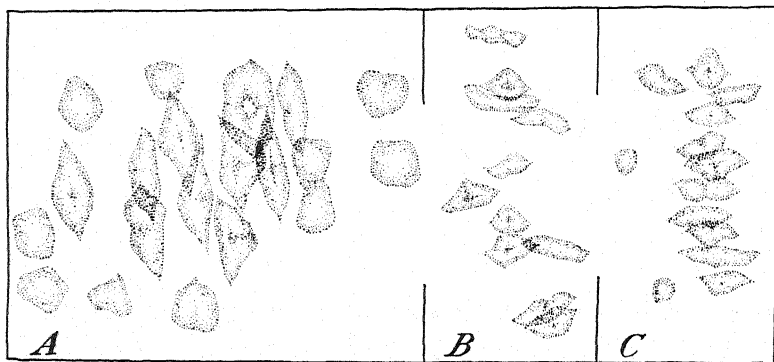
FIGURE 2.— First-division metaphase of teosinte-maize hybrids: *A*, 11 bivalent and 8 univalent chromosomes of an F_1 plant; *B*, 11 bivalent chromosomes of a later hybrid generation; *C*, 10 bivalent and 2 univalent chromosomes from a sister plant of *B*. *A* × 1,800; *B* and *C* × 1,200.

somes in any generation following F_1, for then the number of pairs is not limited to 10. For example, in a plant with 30 chromosomes it is possible to have 15 paired and no unpaired chromosomes, and in such a plant the distribution of the chromosomes at the first meiotic division invariably will be 15 chromosomes to each daughter cell. To calculate an expected chance distribution of chromosomes in 30-chromosome plants of perjugate generations it is necessary first to calculate the expected chance distribution of additional pairs and then apply a binomial distribution to the remaining unpaired chromosomes. Distributions have been calculated for plants with 22, 23, and 24 to 40 chromosomes, based on random distribution of the extra chromosomes from an unlimited number of sets of 10 chromosomes. The observed distributions of chromosomes in many of the plants with a few extra chromosomes are sufficiently close to the calculated distributions to verify the assumption that in later generations the distribution approaches this modified distribution more nearly than it does the simple binomial expansion of the number of chromosomes above 20 present in an individual. However, agreement with such a modified distribution of extra chromosomes does not conflict with

the statement that univalent chromosomes are distributed at random, but is simply a more exact method of estimating the number of unpaired chromosomes.

TABLE 1.—*Distribution of chromosomes to the 2 daughter nuclei resulting from the first meiotic division of 30-chromosome hybrids*

Chromosomes	Number of cells with indicated chromosome distribution					
	15–15	16–14	17–13	18–12	19–11	20–10
Observed	17	30	14	9	5	0
Calculated $(a+b)^{10}$	18.4	30.8	17.6	6.6	1.5	.00+

The data presented in table 1, supported by similar data from drawings of chromosomes of hybrids having chromosome numbers
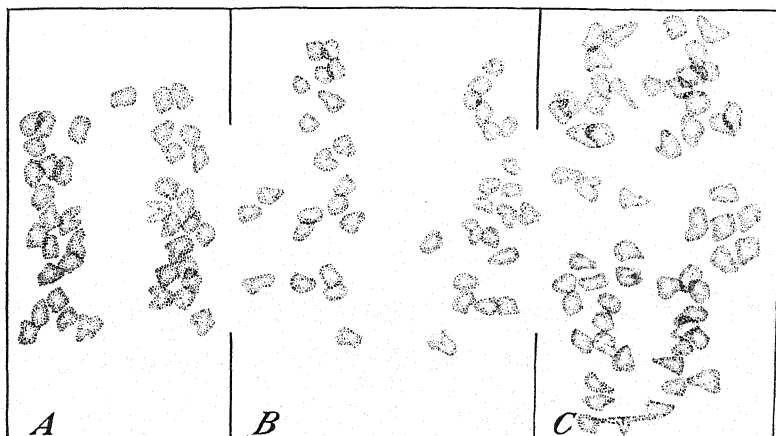
FIGURE 3.—First-division anaphases of teosinte-maize hybrids with aberrant chromosome numbers: *A*, 40 chromosomes from an F_1 plant; *B*, 42 chromosomes from a plant of a progeny twice back-crossed on maize; *C*, 56 chromosomes from an F_2 plant. × 1200

other than 30, indicate that there is a random distribution of all unpaired chromosomes and that gametes are produced with all chromosome numbers in approximately the predicted numbers in each group, the number of chromosome groups depending upon the number of univalent chromosomes present to be distributed at random.

ABERRANT HYBRID PLANTS

In a progeny of 7 F_1 hybrids grown in 1932, it was noticed that 1 plant was markedly different from the 6 sister plants or from any of the previously grown F_1 hybrids. The ears were generally 4-rowed, the pollen showed very little sterility, and abundant seeds were produced after either cross-pollination or selfing. This plant was found to have 40 chromosomes (fig. 3, *A*). The fact that it seemed more cornlike than other F_1 plants suggested that this increase of 10 chromosomes above that of normal F_1 plants was due to the presence of two sets of corn chromosomes. Perhaps the most logical

assumption to account for the double number of chromosomes is that doubling took place soon after fertilization; but other possibilities, such as double fertilization, make it unwise to speculate.

Emerson and Beadle (6) have described a similar aberrant F_1 plant, and the present plant agrees very closely with their description. Such plants, as they suggest, may offer possibilities for genetic and cytological studies.

A small F_2 population from the aberrant plant was grown. The plants were very uniform in appearance, and chromosome counts showed that all of them had 40 or approximately 40 chromosomes.

A second aberrant plant appeared in a progeny twice back-crossed with maize. Sister plants of this progeny were found to have chromosome numbers ranging from 20 to 28, whereas this plant had 42 chromosomes (fig. 3, B). Such a high number suggests a doubling of all chromosomes after fertilization. Although all the experimental plants resulted from guarded pollination, outcrossing might explain the high number of chromosomes; but such an explanation is unlikely, since the plant had 14 more chromosomes than were found in the sister plant having the next highest number. To produce a plant with 42 chromosomes would require a functioning pollen grain with 24 or more chromosomes, a number exceptionally high for the pollen of any hybrid that has yet been grown. The presence of the *wx* gene further reduced the probability that the plant resulted from outcrossing, since no plants were being grown that could produce pollen grains having high numbers of chromosomes and the *wx* gene.

Two plants, one with 56 chromosomes (fig. 3, C), from the F_2 progenies of teosinte and maize, have had high chromosome numbers that suggested a doubling of the chromosomes contributed by either parent or by both. The occurrence of these plants with high chromosome numbers is in keeping with the finding of similar abnormal hybrid plants by investigators of other plant groups.

CHROMOSOME MORPHOLOGY

The investigations of McClintock (16) and Beadle (1) have stimulated an attempt to search the midprophase of some of the hybrid plants, since in many forms there are three allelomorphic chromosomes and often it would be very useful if such chromosomes could be identified. A few plants having only 21 chromosomes are known to be trisomic for chromosome no. 9, the chromosome that carries the *wx* gene, and it seemed that such plants would be useful for a preliminary study. This study, however, has proved disappointing, partly because of the lack of any definite morphological knowledge of the chromosome complement of *Euchlaena perennis*. It has not been difficult to identify chromosome no. 5 (of *Zea*) by its characteristic attachment to the nucleolus. A short chromosome with a terminal knob on the short arm was quite frequently lying where it could be traced and drawn. This chromosome resembles McClintock's drawings of chromosome no. 9, but Beadle (1) has shown that terminal knobs are prevalent on the chromosomes of Florida teosinte. It seems unsafe, therefore, to ascribe a definite identification to such a chromosome in these hybrid plants.

TABLE 2.—*Chromosomes in back-crossed and F_2 Euchlaena perennis–Zea mays hybrids*

Female parent – Species or cross	Female parent – Chromosomes, No.	Male parent – Species or cross	Male parent – Chromosomes, No.	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	Total plants, No.	Mean chromosome number – Observed	Mean chromosome number – Expected	Mean chromosome number – Of functioning gametes of hybrid plant
E. perennis × Z. mays	30	Z. mays	20	1	1	1	1	4	5	5	5	2		1													26	25.3	25	15.3
Z. mays	20	E. perennis × Z. mays	30									4	24	66	8												102	29.8	25	19.8
E. perennis × Z. mays	30	...do...	30				1			1	1			1	1	3	5	14	23	29	31	19	5	5		1	140	35.94	30	

A disconcerting observation of the threads of midprophases was the absence of univalent threads in plants known to have one or often several univalent chromosomes. Beadle (*1*) has referred to a similar absence of univalent threads in hybrids of Florida teosinte and maize. A further study of what seems to be very suitable material is being made in an attempt to definitely trace the behavior of the univalent chromosomes in early prophase stages.

CHROMOSOME SELECTION IN GAMETES

The study of the chromosome numbers prevalent in the functioning pollen and ovules of any particular teosinte-maize hybrid is most readily determined by making reciprocal crosses on maize in which the gametes are known to have 10 chromosomes. By subtracting 10 chromosomes from the chromosome numbers of the plants of the back-crossed progeny, the chromosome number of the gametes of the hybrid parent is obtained. The data of tables 2, 3, and 4 summarize the observed chromosome numbers of the plants of these and other more complicated progenies.

The data of table 2 give the chromosome counts of 26 back-crossed plants in which the F_1 was the female parent, of 102 in which the F_1 was the male parent, and of 140 F_2 plants.

The mean chromosome number of the 26 plants used to determine the chromosome number of the functioning ovules of F_1 plants is 25.3. If 10 is subtracted, the mean number for functioning ovules becomes 15.3, which is just slightly above the expected mean for a 30-chromosome plant. The distribution of these plants among chromosome groups is approximately random.

The mean chromosome number of the 102 plants used to determine the chromosome number of the functioning pollen of F_1 plants is 29.8. If 10 is subtracted, the mean number for functioning pollen becomes 19.8, a number far above the expected mean. The distribution is also very limited, with a range of but 4 chromosomes.

Three plants produced by using F_1 pollen on *Euchlaena perennis* have a mean chromosome number of 39.66, thus showing that the gametes of the F_1 that functioned in their production had 19.66 chromosomes, a number that agrees very closely with 19.8 obtained from the larger population.

It is apparent that the chromosome number of the functioning ovules is in agreement with the chromosome numbers observed going to form the daughter nuclei at the first meiotic divisions. In the pollen, however, there is a marked discrepancy between the observed chromosome distribution at the time the pollen is formed and the chromosome numbers found in functioning pollen. The mean chromosome number, 19.8, shows clearly that there is a tendency for functioning pollen to be restricted to gametes with 20 chromosomes.

In the F_2 progeny the chromosome number ranges from 25 to 42, with a mean number of about 36. This is approximately the mean number expected from combining the mean numbers for functioning ovules and pollen. The distribution approaches that expected from combining the distribution of the ovules and pollen. Three plants, however, with the chromosome numbers 25, 26, and 27, respectively, seem to be sufficiently segregated from the major group to suggest that these three plants were produced from pollen with approximately 10 instead of 20 chromosomes.

TABLE 3.—*Chromosomes in back crosses on Zea mays and selfed back crosses*

Female parent		Male parent		Number of plants with indicated chromosome number																					Total plants	Mean chromosome number		Off-functioning gametes of hybrid parent
Chromosomes	Species or back cross	Chromosomes	Species or back cross	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40		Observed	Expected	
No.		No.		No.																					No.			
30	Z. mays × (E. perennis × Z. mays)	20	Z. mays	8	13	20	32	25	16	10	4	1	2	---	1										132	23.44	25	13.44
20	Z. mays	30	Z. mays × (E. perennis × Z. mays)	6	1					1	2	3	14	13	5	1									47	27.87	25	17.87
30	Z. mays × (E. perennis × Z. mays)	30	do.	4	1	5	3	3	4	2	5	1	5	12	6	14	22	28	25	17	8	10	---	4	179	32.44	30	---

Table 3 presents the data from back-crossed plants that have been back-crossed again onto maize or selfed. The hybrid parents given in this table have 30 chromosomes, and so do not differ in chromosome number from the F_1 plants given in table 2. The chromosome complement has been appreciably changed, however, for instead of the 30 chromosomes being 20 teosinte and 10 maize, as is found in F_1 plants, there will be approximately 10 teosinte and 20 maize chromosomes if autosyndesis has been general.

The mean chromosome number of the functioning ovules of these back-crossed plants (having two sets of maize chromosomes in their chromosome complement), as measured by the chromosome number in plants from back crosses crossed with maize, is 13.44. This mean number is appreciably less than that found for the functioning ovules of F_1 plants and suggests that there is a tendency in this group of hybrids with a predominance of maize chromosomes to develop those ovules with chromosome numbers approximating the haploid chromosome number of maize.

The mean chromosome number of the functioning pollen of 30-chromosome back-crossed plants is 17.87. This number is also appreciably less than that found for F_1 plants. The more striking difference, however, is the bimodal distribution shown by these 47 plants, a distribution that was absent in the 102 plants used to measure the chromosome number of F_1 pollen, although suggested in the discussion of the slight bimodal tendency observed in the chromosome number of F_2 plants. The grouping of the chromosome numbers with one mode at 20.25 and another at 29.2 seems to demonstrate that functioning pollen tends to approach very closely the haploid chromosome numbers of maize or teosinte.

The mean chromosome number of selfed 30-chromosome back-crossed plants is 32.44 which is a little above the sum of the means of the chromosome numbers of the functioning ovules and pollen of back-crossed plants. The difference, however, is not significant, and the distribution of the plants among the various chromosome classes, although too complicated for a complete analysis, suggests two modes—one a little below 25 and the other a little below 35.

Table 4 is made up of additional data for various hybrids. It is apparent that in those progenies in which the female ancestor is the hybrid parent and the male is maize the chromosome numbers of functioning ovules of the female ancestor tend to approach the basic number, 10; but this tendency, although apparent, is sufficiently flexible to allow ovules with any chromosome number between 10 and 20 to function. Some additional data are presented to show the chromosome numbers of pollen of several complicated hybrids. The male ancestors of these progenies, largely maize in their composition, are found to have, in functioning pollen, chromosome numbers approximating the basic number, 10.

In no plant used to show the chromosome numbers of functioning pollen has a gamete been found having 23, 24, or 25 chromosomes—numbers that in many progenies would be expected to predominate. This is a clear indication that the chromosome numbers in functioning pollen are modifications of the numbers that are prevalent in the gametes at the time of their formation. Such modifications apparently result from a differential death rate during development or from a differential growth rate down the pollen tubes, or from both.

TABLE 4.—*Chromosomes in various Euchlaena perennis-Zea mays hybrids*

Female parent — Species or cross	Female parent — Chromosomes No.	Male parent — Species or cross	Male parent — Chromosomes No.	Number of plants with indicated chromosome number																			Plant No.	Mean chromosome number — Actual	Mean chromosome number — Expected	Mean chromosome number — Of functioning gametes of hybrid parent
				20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	No.			
Z. mays × (E. perennis × Z. mays)	29	Z. mays	20		1	3	2	1				1											8	23.12	24.5	13.12
Z. mays	20	Z. mays × (E. perennis × Z. mays)	31		1	3	3	6	1	2	1	1											22	25.82	25.5	15.82
Z. mays × (E. perennis × Z. mays)	28	do.	28									1	2	2	2	4		1					10	29.90	28	—
Do.	29	do.	29			4	3	6	1	2					1	1	2	3					26	26.92	29	—
Do.	31	do.	31									1	1	2	1	1	2	3	2	2		1	19	32.16	31	—
Z. mays × (E. perennis × Z. mays) S. P.	27	Z. mays	20	5	7	7	9	3	2	2													35	22.34	23.5	12.34
Z. mays	33	do. × (E. perennis × Z. mays) S. P. [a]	20			3	11	22	29	26	5	2	1										99	24.93	26.5	14.93
(Z. mays × (E. perennis × Z. mays)	20	Z. mays	27	70	14	1																	85	20.19	23.5	10.19
Z. mays	22	(Z. mays × (E. perennis × Z. mays)	20	25	25	10																	60	20.75	21	10.75
Z. mays × (Z. mays × (E. perennis × Z. mays))	20	Z. mays × (Z. mays)	22	60	1																		61	20.02	21	10.02
Do.	29	Z. mays × (Z. mays × (E. perennis × Z. mays)).	29		2	4	3	2	2	2	1		1	1		1		1		2	1	1	18	24.17	29	—
Z. mays (F2)	31	do.	31									1	1	3	1	2	3	1	2	2	1		17	32.59	31	13.51
Do.	29	Z. mays	20	4	17	10	14	12	5	1													63	23.51	24.5	13.75
Z. mays	20	do.	20		6	6	8	1	12	5	1												24	23.75	25.5	10.12
Z. mays	29	Z. mays (F2)	29	22	3					2	1												25	20.12	24.5	

[a] Selfed progeny.

The data presented in table 4 to show the chromosome numbers prevalent in selfed progenies do not seem to merit extensive analysis, but they do indicate a bimodal tendency that must be due in large measure to the differential functioning of pollen having the basic chromosome numbers of either the maize or the teosinte parents.

DISCUSSION

The foregoing data indicate that in hybrids between *Euchlaena perennis* and *Zea mays* pollen effective in fertilization has a chromosome number approaching the chromosome number of the pollen of either of the ancestral forms, i.e., 10 or 20. In F_1 plants, as measured in back crosses, there is a very marked tendency for pollen with 20 chromosomes to effect fertilization. However, three F_2 plants with low chromosome numbers suggest that pollen with about 10 chromosomes has occasionally functioned.

That pollen grains with 10 chromosomes do, in fact, occasionally function is substantiated by the chromosome counts made in back crosses of various hybrids on corn. The functioning pollen from most back crosses on corn has approximately 20 chromosomes, but one progeny included in the data showed that pollen effective in fertilization approached the two extremes, 10 and 20, in about equal numbers. The pollen from the more cornlike hybrids derived from crossing a back cross onto corn had invariably 10 or approximately 10 chromosomes.

All studies made of many different types of hybrids between teosinte and maize have shown a complete absence of pollen with 13, 14, and 15 chromosomes. The high percentage of empty pollen grains characteristic of the F_1 plants indicates that there has been an elimination of pollen through death early in its development. In other hybrids, however, there is conclusive proof that after pollination there is a differential death or growth rate which eliminates pollen other than that having approximately 10 and 20 chromosomes. This is shown by contrasting the percentage of waxy pollen in mature pollen and the percentage of waxy seeds obtained when this pollen is used to pollinate homozygous waxy plants.

The data accumulated to determine the number of chromosomes in functioning ovules tend to show that ovules are distributed at random among the various classes. The mean chromosome number observed is frequently not a significant departure from the calculated mean. There are, however, in some of the more cornlike progenies significant departures that show a tendency for the selection of gametes with low chromosome numbers. It seems, therefore, that in the ovules there is a slight tendency to favor the survival of the parental chromosome numbers, but the data are too meager to show a bimodal distribution even if it was present. It is evident, however, that the differential survival of gametes possessing parental numbers is much less marked in the ovules than in the pollen.

The effect of the pronounced tendency for euploid gametes to be more virile—a tendency very pronounced in the pollen and suggested in the ovules of teosinte-maize hybrids—is to produce many plants with 20 and 40 chromosomes.

The chromosome complement, however, in these 20- or 40-chromosome plants is profoundly affected by the prevalence of autosyndesis. A hybrid with 2 sets of teosinte chromosomes will produce gametes with 1 set of teosinte chromosomes irrespective of the number

of maize chromosomes present, or if the 2 sets of chromosomes are from maize there will be 1 set of maize chromosomes in all gametes formed. Such a chromosome distribution leads to the presence of 2 sets of teosinte chromosomes in all F_2 plants and 2 sets of maize chromosomes in all plants of selfed progenies from back crosses on maize.

The number of maize chromosomes in F_2 progenies and in back crosses on maize and the number of teosinte chromosomes in selfed progenies of these back crosses depend upon the absence of complete autosyndesis and upon the utilization of gametes with aneuploid or with the diploid chromosome numbers.

Cytologists have frequently reported that in hybrids between parents differing in chromosome number there occurs a differential survival of gametes bearing the parental number of chromosomes.

Täckholm (*21*), in his study of polyploid roses, showed that in the *canina* roses there is a marked tendency to mature pollen with a chromosome number near the basic number, 7. He has shown, on the other hand, that this class of roses makes ovules with only 7 chromosomes less than the somatic number of the plant under investigation. Blackburn and Harrison (*2*) have shown that in a pentaploid rose the functioning pollen has 7 chromosomes and that the egg cells have 28.

The elimination of all pollen except that with 7 chromosomes is in harmony with the selection found in many progenies of *Euchlaena-Zea* hybrids in which only those pollen grains functioned that had the basic chromosome number 10 or approximately 10.

The studies of the distribution of chromosomes in wheat hybrids and the number of chromosomes in functioning gametes is touched upon by Sax (*18*). In a later paper (*19*) he goes more fully into the chromosome number of functioning gametes of a 35-chromosome hybrid and finds that both male and female gametes have 14 chromosomes more frequently than would be expected on the assumption of a random distribution of 7 single chromosomes. Thompson (*22*), and more recently Thompson and Cameron (*23*), find that in a similar wheat cross there is an elimination of gametes with chromosome numbers between the two modes 14 and 21, which is more pronounced in the pollen but significant in the ovules. Watkins (*24*) finds that in a pentaploid wheat hybrid the ovules are generally fertile, whereas in the pollen grains there is a high degree of sterility. He finds further that pollen grains possessing either 14 or 21 chromosomes are more likely to function than those having other numbers of chromosomes.

The random distribution of unpaired chromosomes of wheat hybrids at the time the gametes are formed and the later elimination of gametes with chromosome numbers between the two extremes, as described by the foregoing investigators, are very similar to the conditions found in this study of *Euchlaena-Zea* hybrids. In these hybrids gametes with all chromosome numbers between the two modes are formed, but in the pollen and to a lesser degree in the ovules there is a marked tendency to eliminate all but the low and the high chromosome numbers.

The studies by Goodspeed, Clausen, and Chipman (*10*) and by Goodspeed and Clausen (*9*) of *Nicotiana* hybrids show in some cases that a triploid distributes its 12 univalent chromosomes at random in

the first division and that there is a random survival of pollen in the different chromosome classes at the time of fertilization.

Karpechenko (*12*) has found that viable gametes of F_1 hybrids of *Raphanus* × *Brassica* ($2n = 18$ chromosomes), usually have 18 chromosomes—9 *Raphanus* and 9 *Brassica*. The pollen of F_1 *Euchlaena-Zea* hybrids is similar in constitution to that described by Karpechenko, since in both cases there is reason to believe that the chromosome complement of most of the functioning pollen is made up of one set from each of the parents.

Blakeslee and Farnham (*3*) found that daturas having $2n+1$ chromosomes produce ovules with n, $n+1$, and $n+2$ chromosomes, whereas practically no pollen that functions carries extra chromosomes. McClintock (*15*) has shown that in *Zea* plants which have extra chromosomes there is a decided selection against extra chromosomes in the male gametes and a less obvious selection against eggs carrying extra chromosomes.

It is apparent that selection of gametes with chromosome numbers approaching the basic number or a multiple of the basic number present in the hybrid's ancestors has occurred in widely separated groups of plants. In some cases the gametes have borne the haploid number of chromosomes and in others the diploid number. In most cases it seems that the gametes formed represent all chromosome numbers between the two extremes and that the selection takes place either by differential viability or by differential growth rate. One case, namely, that of the roses, has been described where the formation of ovules is irregular and the eggs lack only 7 chromosomes of the somatic chromosome number characteristic of the hybrid.

Where the chromosome complement of a plant is made up of chromosomes in addition to the two homologous sets, the tendency of the functioning gametes to have the basic chromosome number or a multiple of this number must lead to the production of plants with chromosome numbers in multiples of the basic number and to the absence of plants with aneuploid chromosome numbers.

Indirect demonstration of the selection of gametes is seen in the chromosome numbers of a plant group such as *Rubus* (*8*) or other similar groups which are found to have chromosome numbers only in multiples of the basic number and in which hybridization frequently occurs between forms with different chromosome numbers. The lack of aneuploid forms in such groups seems to be the result of gametic selection favoring the parental numbers.

If triploid hybrids utilized only euploid gametes, three groups of plants, namely, diploid, triploid, and tetraploid, would be found in the offspring.

The tetraploid plants represent a fairly stable group distinct from either parent and contain two sets of chromosomes of the two parents if autosyndesis has been the rule. Darlington (*5*) has stressed the value of such hybrids in productive plant breeding. It is true that in tetraploids there is a proportionate increase in genes with an increase in chromosomes, but a crypt hybrid may breed true for many generations with only rare indications of its hybrid nature. If autosyndesis prevails, a tetraploid may defy our best efforts to segregate and stabilize hidden genetic characters.

Gigantism has frequently been associated with marked increase in chromosome numbers, and consequently tetraploids should be desir-

able in cases where gigantism is a desired factor. Tetraploid peren-
nial teosinte, however, is no larger than the diploid annual form.

Gregory (*11*) and Sinotô (*20*) have demonstrated that gigantism
occurs in forms having few chromosomes as well as in forms having
many. Frost (*7*) and Longley (*14*) have found that tetraploid citrus
plants are dwarfs as compared with their diploid relatives.

It seems to have been rather generally accepted that in isolating
improved strains from polyploid hybrids the progenies with a large
number of chromosomes provide the most desirable material. The
idea may have originated from the cases of gigantism associated with
a doubling of the chromosome number or from the fact that improved
varieties tend to have a higher chromosome number than unimproved
or wild stocks.

It has been pointed out that only a small percentage of tetraploid
forms exhibit gigantism. The two tetraploids produced in the pres-
ent study showed no increased vigor. The increased number of
chromosomes in improved varieties may be accounted for in another
way. Chromosome number is a character which was not considered
in the breeding of existing varieties but which has been observed
since the varieties were developed. If favorable variations occurred
in the same ratio in diploid and tetraploid derivatives but if tetra-
ploid derivatives were more numerous, high chromosome numbers
would predominate in the varieties finally produced. In the de-
scendants of the maize-perennial teosinte hybrids the mean chromo-
some number is well above the mean of the parents, but no correlation
between chromosome number and vigor could be detected.

The final question to consider is that of the bearing of chromosome
number on the recombination of parental characters and the ease
with which desired combinations can be stabilized.

In triploid hybrids where only euploid gametes function, the iso-
lation and stabilization of a new combination of genes is possible
only when allosyndesis occurs in the pairing of the homologous
chromosomes. If autosyndesis always occurs, the tetraploid segre-
gates will combine the parental characters in heterozygous forms and
these forms will reproduce themselves as long as autosyndesis con-
tinues to prevail, but no new homozygous combination of genes is
possible. When allosyndesis occurs, three groups of segregates,
namely, diploid, triploid, and tetraploid, are obtained, all of which
combine the ancestral characters, and from any form containing the
desired combination homozygous plants can eventually be isolated.

Because of the formation of both haploid and diploid gametes, the
triploid segregates are too complex to be discussed here, but the rela-
tive advantage of high and low chromosome numbers may be esti-
mated from diploid and tetraploid derivatives. Assuming that the
triploid hybrid is heterozygous for two simple characters located in
different chromosomes and that is is desired to fix a nonparental
combination of the characters, it follows that:

(1) In the haploid gametes the four possible combinations of
dominants and recessives will be represented in equal numbers, and
in selfed F_2 individuals 1 out of 16 will be homozygous for any desired
combination.

(2) In the diploid gametes there will also be four classes, but none of
them will be pure for nonparental combinations. Three quarters or
nine sixteenths of the selfed F_2 individuals will carry the desired com-

bination but they will not be homozygous; it will therefore be necessary to grow a third or even a fourth generation before the combination can be obtained in a homozygous form.

Consequently it is apparent that a diploid segregate supplies all the parental combinations that may be found in a tetraploid segregate and offers a distinct advantage when an attempt is made to isolate and stabilize any particular combination.

The behavior of F_1 teosinte-maize hybrids has shown that it is possible to obtain from a tetraploid form a diploid form having a chromosome complement made up of 2 of the 4 sets of the tetraploid. From selfing back crosses on maize, plants may be obtained having chromosome complements made up entirely from the chromosomes of the diploid parent.

These findings suggest the possibility that diploid maize may have originated from a hybrid between tetraploid perennial teosinte and some unknown diploid relative. It is much simpler, however, to assume that this hybridization occurred between two diploid forms whose blended characters produced the ancestor to maize, and that *Euchlaena perennis* perhaps does not represent a remnant of a once-prevalent form but rather a recent tetraploid form of the widely distributed annual teosinte.

In the foregoing general discussion of gametic selection in hybrids whose ancestors differed in chromosome number, as well as in the particular discussion of gametic selection in *Euchlaena-Zea* hybrids, the point is emphasized that the selective survival of gametes will profoundly affect the nature of the forms recovered in later generations. The illustrations are not perfect examples of the survival of gametes possessing the basic chromosome number (or multiples of this number) of the form involved. If this tendency were perfect (and it does not seem unreasonable to assume that in many cases it is) its effect on future offspring would be apparent. It seems probable that in nature this differential survival has produced as many recombinations with low as with high chromosome numbers. In this connection the occasional appearance of tetraploids must be cardinal, forming part of an evolutionary process that tends to keep the chromosome number in plant groups of recent origin no higher than those found in the older groups.

SUMMARY

The meiotic behavior of the chromosomes in various teosinte-maize hybrids indicates that homologous chromosomes usually pair and that the unpaired univalent chromosomes are distributed at random to the daughter nuclei. The division of these univalent chromosomes occurs in either the first or the second division.

The regular distribution of all paired chromosomes and the random distribution of all unpaired chromosomes result in gametes with various chromosome numbers ranging from the number of pairs to the number of pairs plus the number of unpaired chromosomes.

The chromosome number of functioning pollen of the various teosinte-maize hybrids is found to approximate either 10 or 20, the haploid chromosome numbers of the two parents, while the chromosome number of functioning ovules shows only a slight tendency toward the euploid numbers, 10 and 20.

An increased viability of euploid gametes seems to be general in hybrids similar to the teosinte-maize hybrids here described, and the survival of gametes with these chromosome numbers profoundly affects the character of the progeny of hybrids.

It is suggested that the diploid derivatives of hybrids offer favorable experimental material.   If allosyndesis has occurred, even the gametes with the haploid chromsome number of the diploid parent will contain chromosomes and consequently characters from both parents.   A diploid derivative will, therefore, combine the ancestral characters of the parents in a plant that seems just as promising for breeding material as those forms with twice as many chromosomes.

The utilization of euploid gametes from tetraploid hybrids restricts the plants of their progenies to three chromosome groups; namely, diploid, triploid, and tetraploid.   The prevalence of diploid forms combining the characters of the ancestors suggests that gametic selection has led to the production of new and eventually stable forms without increasing the chromosome number above that of the diploid parent.

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A BACTERIAL DISEASE OF HEDERA HELIX [1]

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INTRODUCTION

In the spring of 1930 a shipment of English ivy, *Hedera helix* L., was received in New Jersey from Maryland, heavily infected with a bacterial leaf-spot disease. These plants were grown outdoors during the summer, and infections failed to appear on the new growth. Several thousand cuttings were taken from them. In the fall, when these rooted cuttings were potted and grown under greenhouse conditions, the disease reappeared in epidemic form, killing many plants and rendering the remainder worthless. Since a bacterial leaf-spot disease of this host had not previously been mentioned as occurring in the United States, and since under certain conditions it causes serious losses, investigations of its cause and nature were undertaken. The results are reported in this paper.

LITERATURE REVIEW

Lindau (5) [2] in 1894 described a bacterial leaf-spot and stem-canker disease of English ivy in Germany. From his description and illustrations there is no doubt as to the identity of our material and his. No inoculation tests were attempted by Lindau. In his cytological work he failed to find evidence of stomatal infection and concluded that infection took place through the natural wounds on the stem caused by the sloughing off of the pubescence normally present on the tips of young growing stems. Rapidly growing plants were noted as being more susceptible than slower growing plants.

Arnaud (1) in 1920 redescribed the disease from France, giving the name *Bacterium hederae* to the organism he isolated. Although he failed to describe the organism or to report any inoculation trials to prove its pathogenicity, his description of the symptoms again leaves little doubt as to the identity of the disease.

Killian (3) in 1921 reported successful inoculations with *Bacterium hederae* Arnaud, thus proving the pathogenicity of the associated organism. He also described gross characteristics of the organism on several media. The incubation period was determined as from 1 to 3 weeks, depending on the temperature and humidity. He failed to obtain infection on old plant parts, even where wounded.

The first mention of this disease in the United States was made by White [3] in December 1930, followed by rather complete descriptions in August 1931 (8).[4]

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Burkholder and Guterman (*2*) have recently described the same trouble on plants shipped from Georgia to New York and have reported synergism as existing between *Bacterium hederae* and an associated organism also isolated from diseased areas.

ECONOMIC IMPORTANCE

The ivy disease described here has been reported from two commercial nurseries in New York (*2*) and New Jersey, respectively, into which it was imported on plants purchased from growers located in Georgia and Maryland. In 1933 it was found on outdoor-grown ivy in Virginia and in the District of Columbia. Its geographic distribution and importance in the Southern States has not been investigated. Burkholder and Guterman (*2*) give no data on its seriousness in New York.

In New Jersey it immediately became a serious pest in the one large commercial greenhouse into which it was introduced in 1930. Cuttings taken from the stock plants were seriously infected. Over 40,000 were either killed outright or so severely infected that they were discarded as useless. Infections on plants trained into pyramids rendered them unsalable. Under greenhouse conditions where ivy is syringed periodically the spread of the disease is rapid, owing to the dissemination of the bacteria from infected leaf areas and stem cankers by the water. Introduced on *Hedera helix*, this disease has spread to several of its horticultural varieties being grown by this same concern and has persisted and caused injury and losses in spite of all efforts to check it.

SYMPTOMS

ON LEAVES

Recent infections on young leaves become evident in from 5 to 12 days as small translucent, roughly circular spots (pl. 1, *B*). In the earliest stages these spots are very difficult to see except by transmitted light. As the spots enlarge, the center becomes brown to brownish black, dries out, and frequently cracks (pls. 1, *A*, and 2, *A*). Under conditions of high humidity an orange-red bacterial exudate may occur on the infected areas. The older spots are usually surrounded by a light yellowish-green water-soaked area, but on old foliage this may be replaced by a reddish to reddish-brown irregular or scalloped region. Infected areas on the leaves are frequently secondarily infected with either *Colletotrichum trichellum* (Fr.) Duke (pl. 1, *D*) or *Phyllosticta hedericola* Dur. and Mont. This succession of *P. hedericola* following bacterial infections has previously been reported by Nicolas and Aggery (*6*) on *Aralia japonica*.

Infection frequently takes place on or very close to a vein. Under such conditions the spots developing are not circular but are elongated in the direction of the vein, indicating possible systematic invasion (pl. 1, *C*). Heavily infected leaves usually turn yellow and fall.

ON PETIOLES

Direct infection of petioles is rare. The spots that develop from such infections are dark brown to black and enlarge rapidly in both directions and soon girdle the petiole, and the attached leaf wilts (pl. 2, *B*, *F*). Petioles frequently become infected from heavily
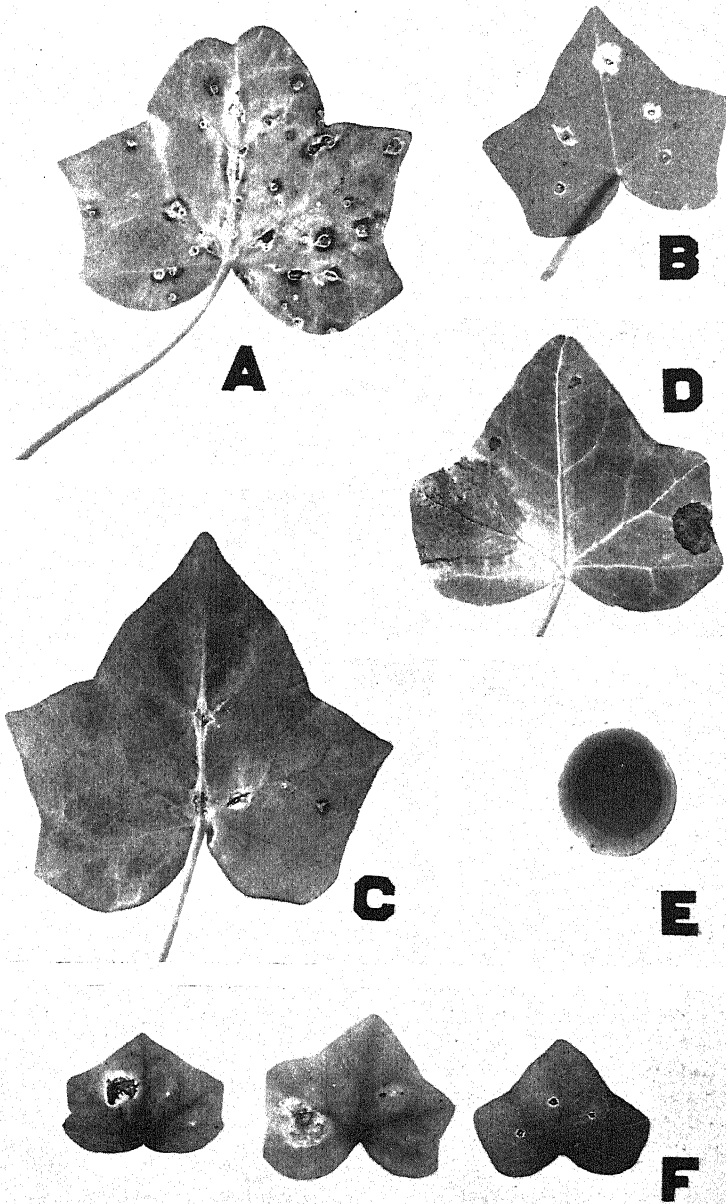
A, Artificial inoculations obtained by brushing bacterial suspension on leaf with camel's-hair brush; B, needle-prick inoculations on young leaf after 8 days; C, artificial inoculations obtained by needle pricks in veins; D, leaf with four natural infections with *Bacterium hederae*, one of which has been followed by *Colletotrichum trichellum;* E, colony of *Bact. hederae* on beef-extract agar; F, leaf on left inoculated with *Bact. hederae;* center, double inoculation with *Bact. hederae* and culture *d;* right, double inoculation with *Bact. hederae* and culture *k;* all 4 weeks after inoculation.

PLATE 2
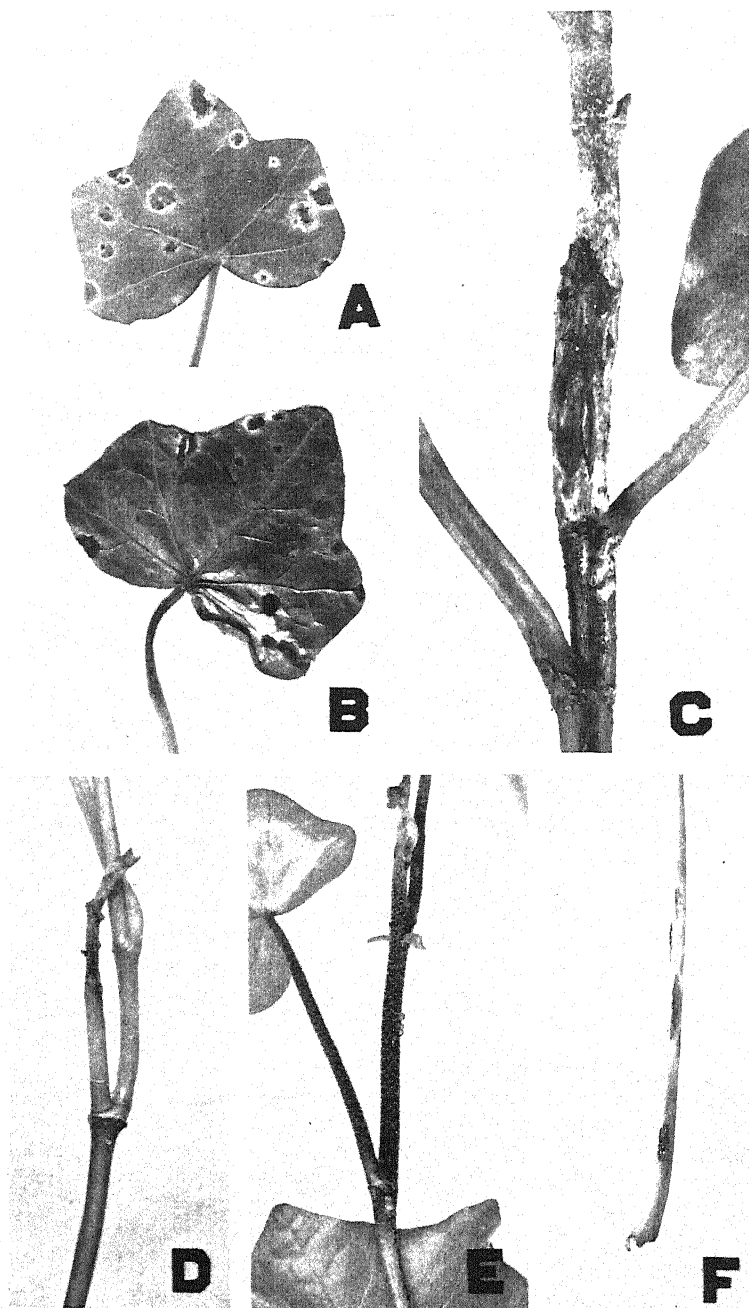
A, Natural leaf infections; B, natural leaf infections and petiole lesions; C, stem canker; D, infection at growing tip; E, bacterial exudate from very young stem canker; F, petiole lesions inoculated by bacterial suspensions brushed on petiole with camel's-hair brush.

infected leaves, however, the bacteria advancing rapidly down the petioles to the stem.

ON STEMS

Infection on stems takes place naturally either from infected petioles or on the very young and tender growing tip (pl. 2, *D*). On young tissues a soft dark-brown to black decay rapidly takes place. Invasion is retarded when older tissue is reached, a new growing point is developed from the next lowest axillary bud, and no further advance is made.

On older tissues of the stem, where infection arises from petioles, definite cankers are produced. At first these cankers appear as small brown sunken areas. Invasion of tissue is slow. Old cankers are flattened and shrunken, usually cracked longitudinally, and surrounded by swollen margins due to callus formation on the part of the host (pl. 2, *C*). Stem cankers on old tissues have never been observed entirely girdling the stem; however, they frequently cause a cessation or retardation of growth and an abnormal light-green coloration of the foliage. Frequently the foliage of plants carrying stem cankers develops a reddish-bronze coloration typical of that produced by maturity in the fall. An orange-red bacterial exudate frequently occurs on these stem cankers (pl. 2, *E*).

VARIETAL SUSCEPTIBILITY

Arnaud (*1*) noticed that of two ivies under observation, one, "Lierre des Bois", was more severely attacked than the other, "Lierre d'Ecosse." The disease has been observed occurring naturally on *Hedera helix* and its varieties *baltica, gracilis, lucida, digitata,* and Silver Queen. Inoculations upon these varieties as well as upon the varieties *marmorata, alba variegata, dentata variegata, conglomerata, nigra,* and *coriacea* have shown all to be susceptible.

PATHOGENICITY

The pathogenicity of the organism constantly associated with these disease symptoms has been repeatedly proved by inoculations in various parts of the host under varied conditions and by various methods. Repeated reisolations and reinoculations have been successfully made. Pure cultures are easily obtained by the usual poured-plate method. Young leaf tissues (plants kept under bell jars in a constantly moist atmosphere) are readily infected by atomizing with bacterial suspensions and show positive results in as short a time as 4 days. The tiny translucent spots appeared in 5 to 6 days, and some were 7 to 8 mm in diameter in 9 days. These lesions were in all respects similar to those found on naturally infected plants. Mature leaves or woody stems show symptoms only after longer periods, varying from 2 to 3 weeks. Such tissues are rarely infected except through wounds.

THE PATHOGENE

The cultures used for the morphological, cultural, and physiological studies were isolated from characteristic leaf lesions. Some of the lesions were the result of natural infections; others were the result of artificial inoculation with the bacteria. The pathogenicity of the several cultures used in these studies was established by successful

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infections induced by inoculation of healthy, growing ivy leaves and stems.

MORPHOLOGY

Bacterium hederae is a short rod with rounded ends, rather smaller than is usual for plant pathogenes. In culture media the rods are 0.7 to 2.7μ long by 0.3 to 0.6μ wide and occur singly or in pairs or short chains. In the host tissues they are 0.7 to 2μ long by 0.2 to 0.4μ wide. They are motile by means of one polar flagellum. Capsules are present. No spores have been found.

STAINING REACTIONS

The organism is Gram-negative; it is not acid-fast. It stains readily with all the commonly used bacteriological stains. The capsules are easily demonstrated with Ribbert's dahlia capsule stain and also with Leifson's (*4*) stain, a flagella stain which stained the capsules but only rarely the flagella of the ivy bacteria which proved to be unusually difficult to stain. However, with Casares-Gil's stain it was definitely determined that there is a single polar flagellum, usually long and often quite wavy in the part nearest the rod.

Carbol-fuchsin-stained mounts from potato-dextrose agar cultures grown at 34° C. showed single rods and chains very poorly and irregularly stained, with diameters varying from 0.3 to 0.9μ. In beef-media cultures the diameter of the bacteria is slightly greater than in potato-dextrose agar cultures.

CULTURAL CHARACTERS[5]

BEEF-PEPTONE AGAR COLONIES.—On beef-peptone agar (pH 6.8 to 7.0) the colonies of *Bacterium hederae* grow slowly. In 48 hours they are usually visible as mere points of growth, and in 4 to 5 days even well-isolated colonies are only 1 to 2 mm in diameter. In 10 to 12 days a few colonies are 5 to 7 mm in diameter, but the usual size is 2 to 4 mm. White and transparent at first, they become pale yellow, Massicot yellow,[6] and translucent. They are circular, smooth, glistening, slightly elevated, with entire margins (pl. 1, *E*). The interior has definite short concentric lines or crosshatching, which disappears when the colonies are 10 to 12 days old. Beef is not a very favorable medium for this organism, and the colony characters vary with even slight differences in the media, the acidity, the moisture, the temperature, or other factors. In one set of plates the colonies had definite white halos. In another set from 3 to 20 points of secondary growth appeared within each colony. Sometimes the centers are opaque with translucent crosshatched borders, or they may be granular, mottled, or homogeneous. Buried colonies are spherical to lenticular, opaque to translucent. The growth is slightly viscid. Another fact noted is that growth in transfers from beef-media cultures is very uncertain. Unless the beef culture is young and rather heavy inoculations are made from it, more often than not no growth develops in the transfers.

BEEF-PEPTONE AGAR SLANTS.—Growth is slow and never becomes even moderately heavy. If the inoculation is from a liquid culture the resulting growth is most likely to be in the form of tiny isolated colonies, which eventually may coalesce. Inoculation from agar cultures gives a uniform, smooth, glistening streak, practically colorless except on the lower part of the slant, where the growth is somewhat thicker and pale yellow. Crosshatching or striae are present. The growth is slightly viscid or elastic; it does not draw out in a long thread but breaks at a length of 4 to 6 mm.

BEEF-EXTRACT AGAR.—Growth is very similar to that in beef-infusion agar, but the color is slightly deeper yellow and the growth is not viscid.

BEEF-PEPTONE BROTH.—The bacteria grow slowly in beef broth, even of most favorable pH value and at favorable temperatures. Thin, rolling clouds, best in

the upper layers of the liquid, appear in 2 days. Growth increases slowly for a number of days, but the clouding is never more than moderate. Irregular rims of pale yellow appear in 5 to 7 days and often become fairly wide and heavy. If cultures are undisturbed a thin pellicle forms. There is a moderate, translucent sediment, which rises in a spiral when shaken. Rims, pellicles, and sediment are viscid. Clouding persists for 5 to 6 months at room temperature.

POTATO-DEXTROSE BROTH.—In this medium, with a pH value of 5.6 to 5.8 the liquid clouds very slightly, but the surface growth in the form of rim and pellicle is heavy.

POTATO-DEXTROSE AGAR.—This medium with a pH value of 5.6 to 5.8 seems to be ideal for the ivy bacterium. In isolation plates the colonies reach a diameter of 10 to 12 mm. In tubes of slanted medium the surface is quickly covered with a thick, smooth, glistening layer of homogeneous to indefinitely mottled (under a $\times$ 6 lens) growth. The growth, practically colorless at first, becomes pale green-ish yellow, Massicot yellow, or Chartreuse, and later chamois [7] or even darker. The texture of the growth is soft and butyrous. Old cultures sometimes show a trace of viscidity. The thick, smooth, translucent growth remains practically unchanged for weeks. The pH of the medium of cultures changes in 3 days from 5.6 to 6.6.

POTATO CYLINDERS.—On steamed potato cylinders growth at first is fairly smooth and pale yellow, but it soon becomes thin, wet, and yellowish brown with some pale yellow at the margin. The potato is moderately browned. Growth does not persist or increase. A weak diastatic action was indicated by tests with Lugol's iodine solution.

BEEF GELATIN.—In plates of beef gelatin with a pH of 7.3, the colonies were barely visible in 2 days at 20° to 23° C. In 5 days the gelatin immediately below the colonies was liquefied. In 7 days the medium of thickly sown plates was entirely liquefied and the small, spherical, compact colonies were floating in the unclouded liquid. In tube cultures slight liquefaction is evident in 2 days. In 6 to 7 days there is a 5- to 7-mm stratiform layer of liquid. Further advance is slower, 5 to 6 weeks being required for liquefaction of the entire 10 cc of medium. The liquefied gelatin is almost entirely clear. Irregular rims and thin pellicles of pale yellow form, and there is a moderate viscid sediment, deeper yellow and more opaque than in beef-broth cultures.

BLOOD SERUM.—On Loeffler's solidified blood serum, growth was doubtful for several days. Later the whole slant was covered with a smooth layer of mustard-yellow growth. No trace of liquefaction was observed until after 4 weeks, when the slanted part became translucent and yellowish. In 7 weeks some cultures were entirely liquefied, while in others only the slanted portion was liquefied. Cultures were now getting dry, and no further change occurred.

REDUCTION OF NITRATES.—Growth is fairly good in nitrate-beef bouillon, but 9-day-old cultures with the starch-iodine-sulphuric acid test showed no trace of nitrate reduction. Cultures in a synthetic nitrate medium tested when 10 days old with the α-naphthylamine sulphanilic-acid as recommended in the Manual of Methods (7), gave positive indications of a moderate reduction of nitrate, less than half as much as in control cultures of *Bacillus phytophthorus* and *B. aroideae*.

DIASTATIC ACTION.—Plates of beef agar plus 0.2 percent of starch were heavily inoculated with surface streaks. Growth was not vigorous. On the eighth and tenth days a partially cleared zone 15 to 20 mm wide appeared when the plates were flooded with iodine solution. Potato-cylinder cultures 5 weeks old tested with iodine also gave indication that the starch is only partially hydrolyzed.

COHN'S SOLUTION.—Repeated trials with light and with heavy inoculations show that the organism does not grow in this medium.

USCHINSKY'S SOLUTION.—When heavily inoculated a slight milky color and thin clouding appeared after 6 to 10 days. In 4 to 6 weeks the clouding was moderate and fairly heavy and pale yellow rims and pellicles formed. There was no color change in the medium.

FERMI'S SOLUTION.—Growth is slow and slight in this medium. Heavily inoculated cultures 3 weeks old are faintly clouded and have a few slender white threads suspended in the liquid or attached to the tube wall. There is no rim of pellicle and no color change in the medium.

TOLERATION OF SODIUM CHLORIDE.—In beef broth containing 1 percent of NaCl, growth is as good as in plain broth. Growth is slightly retarded by 2 percent, greatly retarded by 3 percent, and entirely lacking in 4 percent of NaCl.

INDOL PRODUCTION.—Indol is not produced. Cultures in a 2-percent peptone solution and in 1-percent tryptophane solution grow better than in beef broth.

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[7] RIDGWAY, R. See footnote 6.

The tests were made with sulphuric acid and sodium nitrite. Control cultures of *Bacillus coli* produced indol.

HYDROGEN SULPHIDE PRODUCTION.—A slight amount of hydrogen sulphide is produced. Tests were made in lead acetate agar; also by strips of lead acetate paper suspended over cultures.

AMMONIA PRODUCTION.—Slight amounts of ammonia are produced in beef media and in peptone-broth cultures.

MILK.—Milk is slowly coagulated. The curd remains soft and jellylike for 2 to 3 weeks, then becomes more compact. Casein is slowly digested, about 3 months being required for complete digestion. The whey is yellowish and viscid. Numerous tyrosin crystals form in all milk cultures.

LITMUS REDUCTION.—Lavender-colored litmus-milk shows slight to no bluing. Reduction of the litmus begins in 4 to 8 days and is complete in 6 to 12 days.

METHYLENE BLUE REDUCTION.—Methylene blue in milk is considerably reduced in 2 and entirely reduced in 8 days.

FERMENTATION OF CARBOHYDRATES.—The ability of the bacteria to ferment carbohydrates was tested on peptone-free synthetic agar (7), with brom-cresol purple as an indicator. One percent each of dextrose, sucrose, lactose, maltose, mannite, and glycerin was used. Acid without gas was formed very promptly from dextrose and sucrose, rather slowly from lactose, and very slowly from glycerin. In maltose there was only a trace of growth and no acid reaction. There was no growth in mannite.

TEMPERATURE RELATIONS.—The optimum temperature for growth is between 20° and 26° C. Beef-bouillon cultures cloud more readily at 25° to 26° than at 20° to 22°, but after several days the cultures at the lower temperatures have the better growth. The minimum temperature for growth is 2° or lower. The maximum for beef-bouillon cultures is 32°. (Beef-bouillon cultures cloud thinly at 33° and 34° in 1 to 2 days, but this clouding disappears in 1 day or less.) Slight but persistent growth occurs on beef agar at 34° and on potato-dextrose agar at 35° and 36°. No growth occurred at 37°. The thermal death point is near 52°.

EFFECT OF FREEZING.—Potato-dextrose agar cultures held at 4° to 8° F. (−20° to −22° C.) for 4½ months, except for several short intervals of partial or complete thawing, were not killed or even noticeably reduced in vitality.

EFFECT OF SUNLIGHT.—Freshly inoculated plates of beef agar with half of each plate covered to exclude direct light were exposed for 10, 20, and 30 minutes to direct sunlight at midday, April 5, 1932. A slight haze but no clouds slightly reduced the light. In these tests the bacteria were killed in the areas subjected to the direct rays of the sun, and also to a considerable distance under the covered parts. Colony numbers were normal only in the area farthest from the light. In a repetition of the experiment at midday, April 12, 1932, when there was no haze, all bacteria were killed by exposure for 10 minutes; in 6 minutes 80 to 90 percent were killed, and in 3 minutes 40 to 60 percent were killed.

OPTIMUM REACTION AND TOLERATION LIMITS.—Because of the slow growth of the bacteria the pH values of the medium may change somewhat before the cultures cloud. Repeated tests, with the pH values of the media determined at inoculation time and again when growth became evident, indicate 7.0 as the optimum for growth, and 5.5 and 8.5 as the limits. The different strains studied varied slightly, and at different times the same strain sometimes showed slight variations in the pH requirements.

RELATION TO OXYGEN.—The organism is aerobic. There was no clouding in the closed ends of fermentation tubes containing beef bouillon and synthetic media plus carbohydrates, nor in agar tubes either with or without carbohydrates.

VITALITY IN CULTURE.—In culture media, particularly in beef media, the vitality of the ivy bacterium shows a lack of uniformity. At room temperature some beef cultures have remained alive for 7 months, but most cultures die within 2 to 8 weeks. Many transfers to beef media, especially if made from liquid culture, fail to establish any growth. On potato-dextrose agar there is no difficulty in securing vigorous growth, and no reduction in vitality has been noted in cultures of various ages up to and including some 9 months old. At temperatures below freezing, the organism remains alive at least 4 months on potato-dextrose agar. Cultures that grew slightly at 34° and 35° C. and remained at these temperatures for 20 to 30 days were to some extent reduced in vitality, as shown by growth in transfers under favorable conditions but by failure to grow under slightly adverse conditions.

EFFECT OF DESICCATION.—Tests for resistance to drying were made by placing drops from beef-bouillon cultures or growth from agar cultures diluted in water or broth on cover glasses, and after these had dried, introducing them at intervals

into culture media. As in transfers from beef cultures, there was a lack of uniformity in growth results. A large number of covers failed to give any growth after drying even a few days. Some, however, produced typical growth after drying for 43 days. If the covers were put into beef bouillon, growth very seldom developed. The best method found was to embed the cover partially in very moist potato-dextrose agar.

HISTOLOGY

Thin, stained sections of ivy leaves collected 9 days after inoculation by spraying with suspensions of *Bacterium hederae* show numerous small infected areas on the lower side of the leaf. Bacteria are abundant in these lesions, most of which have penetrated only a short distance; others extend through half the thickness of the leaf and spread laterally an equal distance. In these sections the lesions are advanced to a stage where the lower epidermal cells are broken or distorted, and cases of distinct stomatal infection have not been seen. Stomata are present on the lower surface only of the ivy, and since infection starts on the lower side of leaves inoculated by spraying, it seems more than probable that the infection is stomatal. The numerous and large intercellular spaces in these leaves afford good accommodations for masses of bacteria. First the bacteria occupy the intercellular spaces; later the cell walls break. In some cases the bacteria seem to be inside intact cell walls. In sections of older lesions there is considerable breaking down of cell walls, the bacteria occupy large pockets, and the upper as well as the lower epidermis is destroyed. Sections of leaves collected 48 hours after inoculation show no sign of infection. The bacteria in the lesions are 0.7 to 2μ by 0.2 to 0.4μ wide. Capsules were stained on rods direct from leaf lesions. Staining of flagella on leaf-lesion rods was not attempted.

TECHNICAL DESCRIPTION

Bacterium hederae Arnaud is a short, motile rod, 0.7 to 2.7μ by 0.3 to 0.6μ, with a single polar flagellum. Capsules are produced, but no spores. It is Gram-negative and is not acid-fast. It is aerobic. Gelatin and blood serum are slowly liquefied. Nitrate is slightly reduced, but no gas is produced. Diastatic action is slight. Acid without gas is formed from dextrose, sucrose, lactose, and glycerin. The organism does not form acid in milk, but reduces litmus and methylene blue in this medium. Indol is not formed. Slight amounts of hydrogen sulphide and ammonia are produced. Its optimum pH value for growth on beef media is 7.0, and the optimum temperature for growth is 20° to 26° C., maximum 36°, minimum below 2°. Its thermal death point is 52°. On beef agar growth is moderate to slight. Colonies are round, smooth, pale yellow. On potato-dextrose agar growth is abundant, pale yellow. It causes leaf spots and stem cankers on English ivy, *Hedera helix*, and its horticultural varieties.

SYNERGISM

With the appearance of Burkholder and Guterman's (*2*) report of synergism between *Bacterium hederae* and an associated organism, experiments were conducted with 10 associated bacteria which the present writers had isolated and designated as cultures *d* to *m*. Without exception these associated organisms were isolated from old spots on foliage. Isolations from young diseased areas never failed to yield pure cultures of *Bact. hederae*.

Inoculations were made on leaves injured by needle pricks with *Bacterium hederae* in pure culture and also mixed with each of the 10 associated bacteria. In one case when culture *d* was mixed with *Bact. hederae* a distinct increase in size of the infected area resulted, which was still evident after 4 weeks from the time of inoculation. In all other nine mixed inoculations (*Bact. hederae* plus cultures *e–m*), the exact reverse process took place, or a case of anergism. The spots resulting from these mixed inoculations after 1 month were approximately 2 mm in diameter and lacked the water-soaked margins characteristic of active invasion of tissue. Inoculations with *Bact. hederae* in pure culture produced spots 7 mm in diameter, and with *Bact. hederae* and culture *d* 12 mm in diameter in the same time (pl. 1, *F*).

Organisms *d* and *k* are not the associated organisms reported by Burkholder and Guterman (*2*). Their identity is unknown. The anergistic action of nine other associated organisms (*e* to *m*, inclusive) is worthy of note.

CONTROL MEASURES

As measures of control of this disease, all soil, sand, or cinders on which an infected lot of plants have stood should be removed and the beds or benches sterilized by washing or spraying with formaldehyde solution 1:50 or corrosive sublimate 1:1,000, before placing another lot of potted cuttings on them. If the infection on any lot of plants is slight, hand-picking of the infected foliage, the pruning out of all tip infections, and the discarding of plants showing stem cankers can be resorted to and will lessen the danger of increasing severity. Keeping the temperatures of the houses at 50° F. or below has also seemed to check the rapidity of spread. Excessive syringing of the plants should be avoided, as this practice tends to spread the disease, particularly from plant to plant in the same bench.

The use of protective sprays on ivy is not desirable, because of the residue deposited by them on the foliage. Potassium permanganate has been tried in concentrations as high as 1:600. At this concentration some injury took place, but injury was absent at a concentration of 1:800. The grower felt that considerable advantage from the standpoint of disease control was obtained after repeated applications, although no unsprayed plants were held as checks against these sprays. Mercuric bichloride at a concentration of 1:1,000 has also been used, and this caused slight injury to the young foliage.

Preliminary tests were made with proprietary organic-mercury sprays containing as the toxic ingredient ethyl mercury arsenate and phenyl mercury acetate in concentrations of 1:600 to 1:50. A single application of these materials at any of the concentrations used caused no perceptible injury, but with subsequent applications the foliage became yellowish and in some cases the young foliage became curled and crinkled. As a result of this injury these materials were not used in further control tests.

SUMMARY

A bacterial disease of English ivy, *Hedera helix* L., causing a leaf spot and stem canker is described. From the results of inoculations on 12 horticultural varieties, all were found susceptible. The incubation period varied from 5 to 21 days, depending on the temperature,

humidity, and age and type of tissues inoculated. Young tissues are more easily infected than old ones, and the incubation period is proportionately shorter.

The causal organism (*Bacterium hederae* Arnaud) has been isolated and its pathogenicity proved by numerous inoculation experiments. Wounds are not necessary for infection, which is evidently stomatal.

The morphological, cultural, and physiological studies of the pathogene are described in detail and a technical description is given. The index number is 5322–3115–1222.

Synergism with 1 associated bacterial organism and anergism with 9 other associated bacterial organisms were noted.

Various methods of control are suggested.

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GROWTH OF CHICKENS AS A FUNCTION OF FEED CONSUMPTION [1]

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INTRODUCTION

The relationship between the weight of feed consumed by a growing animal and the resulting gain in live weight is one of both practical importance and theoretical interest. As a result of a large number of scattered observations, much information has been obtained regarding the factors which influence the economy of gains made by domestic animals, but the information so obtained has never been fully organized. Students of animal nutrition have realized for a long time the need of more exact knowledge of this kind; nevertheless it was not until about 10 years ago, when Spillman (*4*) [2] suggested the use of the equation of the curve of diminishing increment for express-ing the relationship between feed consumption and live weight, that, from the point of view of the present writers, the first real advance was made.

In previous papers Jull and Titus (*3*) and Titus (*5*) showed that in crossbred chickens and ducks the equation of the curve of dimin-ishing increment expresses with a high degree of accuracy the rela-tionship between feed consumption and live weight over an appreciable interval of growth. The data presented in these earlier papers, however, did not constitute a rigorous test of the relationship because the same diet was not fed from the first feeding until the conclusion of the experiment, and because a sufficiently long interval of growth was not investigated. Furthermore, as a result of the small number of data involved and the lack of a wholly suitable method of fitting the equation, considerable doubt remained as to the true significance of some of the parameters of the equation.

The experiment described in the present paper was planned for the purpose of (1) obtaining much more extensive and critical data, (2) studying the effect of sex on the utilization of feed, and (3) deter-mining the effect of different levels of feed intake on the relationship between feed consumption and live weight. The plan of the experi-ment provided for (1) feeding the same diet, but at different graduated levels of intake, to 7 pens of males, as well as to 7 pens of females, for a period of 52 weeks; (2) collecting the pertinent data on feed consumption, live weight, and mortality; and (3) making a mathe-matical analysis of the data.

EXPERIMENTAL MATERIAL AND METHODS

Approximately 560 chicks were hatched in electric incubators February 13, 1930, at the United States Animal Husbandry Experi-ment Farm, Beltsville, Md. The eggs were obtained from a flock of

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Barred Plymouth Rock females mated to Rhode Island Red males. This mating was employed in order to make use of the sex-linked barring factor which enables one to separate the sexes at hatching time. After removal of the weak and otherwise unsuitable chicks, there remained 265 males and 244 females. These were distributed among 14 pens so that there were 7 pens containing 37 males each and 7 pens containing 34 females each; the remaining chicks were discarded.

The chicks were brooded under electrically heated brooders in a series of pens in a hot-water-heated brooder house. After they were a few days old they were allowed the freedom of small run yards adjoining the pens. By means of this arrangement the chicks had access to direct sunlight whenever the weather permitted. The floors of the pens and run yards were constructed of concrete.

Approximately 10 percent of the chicks developed perosis (*7, 8*) or other abnormalities and were killed as soon as such abnormalities were observed. During the course of the experiment, as the chicks became larger, others were removed to prevent crowding in the pens.

THE DIET

The following diet, to which 1.5 percent of cod-liver oil was added, was fed in the form of a dry mash to all 14 pens of chicks from the first feeding until the end of the experiment.

Percent

Yellow corn meal	40. 0
Ground wheat	22. 0
Corn gluten meal	10. 0
Dried buttermilk	10. 0
Meat scraps (55 percent protein)	10. 0
Special steamed bone meal	3. 0
Alfalfa-leaf meal	2. 5
Yeast preparation [3]	2. 0
Common salt	. 5
Total	100. 0

This diet, as the average of a number of analyses of different lots of it showed, contained approximately 10.4 percent of moisture, 7.1 percent of ash, 4.9 percent of ether-extractable material, 19.4 percent of crude protein, 2.6 percent of crude fiber, and 55.6 percent of nitrogen-free extract.

LEVELS OF FEED INTAKE

One pen of males and 1 pen of females were given all the feed they would eat, and the other 6 pens of each sex were fed at different lower levels of feed intake. Accurate feed-consumption data obtained over an extended period were not available for this particular crossbreed of chickens. Accordingly, in order to determine how much feed to give to each of the 12 pens of chicks that were to be kept on the lower levels of feed intake, use was made of data previously obtained by the writers with several groups of Rhode Island Red chicks in which the sexes had not been separated. From the average ad libitum feed consumption of these chicks, tables were prepared which gave the weight of feed, per chick, for each day in order that the relative levels of feed intake for the 6 pens of each sex would be

[3] A commercial yeast preparation made by drying a suspension of yeast on corn meal; it contains approximately 30 percent, by weight, of dried yeast.

87.5, 75.0, 62.5, 50.0, 37.5, and 25.0 percent, respectively, of this arbitrarily chosen standard.

The quantity of feed consumed, per chick, by the pen of females which was allowed to eat all the feed that it desired was approximately equal to the ad libitum feed consumption of the Rhode Island Red chicks mentioned previously, but the quantity consumed, per chick, by the corresponding pen of males was appreciably greater. Thus, although the relative levels of feed intake (table 1) were not the same for the corresponding pens of males and females, the absolute levels (actual quantity) of feed intake were practically the same, except for the pen of males and the pen of females which were given all they would consume.

TABLE 1.—*Levels of feed intake of the 7 pens of males and the 7 pens of females*

	Males			Females	
Pen no.	Level of feed intake as percentage of the average ad libitum feed consumption of Rhode Island Reds [a]	Relative level of feed intake, as percentage [b] of the average ad libitum feed consumption of crossbred males	Pen no.	Level of feed intake as percentage of the average ad libitum feed consumption of Rhode Island Reds [a]	Relative level of feed intake, as percentage [b] of the average ad libitum feed consumption of crossbred females
	Percent	*Percent*		*Percent*	*Percent*
84	127.5	100.0	85	102.2	100.0
86	87.5	68.6	87	87.5	85.4
88	75.0	58.9	89	75.0	73.5
90	62.5	49.0	91	62.5	60.9
92	50.0	39.2	93	50.0	49.2
94	37.5	29.4	95	37.5	36.8
96	25.0	19.6	97	25.0	24.5

[a] Sexes not separated.
[b] Levels of feed intake were computed each week, and the averages for the first 36 weeks were taken.

The females in pens 85, 87, 89, and 91 began laying during the twentieth, twenty-third, twenty-fourth, and twenty-seventh weeks, respectively, of the experiment, and for a time an attempt was made to "correct" the feed consumption by subtracting 40 grams of feed for each egg laid (6). By the thirty-sixth week the egg production had increased to such an extent in pens 85, 87, and 89 that the writers believed it was no longer possible to "correct" the feed consumption with a sufficient degree of accuracy, and so these three pens were discontinued at the end of the thirty-sixth week.

EXPERIMENTAL DATA

Records were kept of the quantities of feed consumed per chick per week, of the live weights of the chicks at the end of each week, and of the mortality in each pen.

The experimental data on feed consumption and live weights are too voluminous for presentation here. However, the discussion centers around, and most of the conclusions depend on, a relatively small number of constants derived from the data. These constants are given in the discussion which follows.

Since mortality data are of considerable importance in interpreting the results of feeding experiments, table 2 is given. In general the

number of birds that died was very small except in the pens receiving less than 50 percent of the ad libitum level of feed.

TABLE 2.—*Number of deaths [a] occurring in each pen during each 4-week period*

MALES

Pen no.	Number of deaths during—													Total [b] number of deaths
	First 4 weeks	Second 4 weeks	Third 4 weeks	Fourth 4 weeks	Fifth 4 weeks	Sixth 4 weeks	Seventh 4 weeks	Eighth 4 weeks	Ninth 4 weeks	Tenth 4 weeks	Eleventh 4 weeks	Twelfth 4 weeks	Thirteenth 4 weeks	
84	0	0	0	0	1	0	0	0	0	0	0	0	0	1
86	1	0	0	0	0	0	0	0	1	0	0	0	0	2
88	1	0	0	0	0	0	0	0	0	0	0	0	1	2
90	2	0	0	0	0	0	0	0	0	2	2	0	1	7
92	0	0	0	1	0	0	0	0	3	3	2	2	0	17
94	3	1	0	0	0	1	0	0	1	6	3	0	0	21
96	17	1	0	5	2	2	3	2	2	[c] 1				35

FEMALES

85	0	0	0	2	0	0	0	0	0	([d])				2
87	0	0	0	0	0	0	0	0	1	([d])				1
89	1	0	0	0	0	0	0	0	1	0	([d])			2
91	1	0	0	1	0	0	0	0	0	0	0	0	0	2
93	1	0	0	0	0	2	0	0	0	0	1	0	0	4
95	0	0	1	0	0	0	0	0	3	2	1	2	1	10
97	9	0	0	2	0	2	1	1	7	[e] 7				29

[a] Exclusive of perosis, which does not directly affect growth.
[b] There were 37 chicks in each pen of males and 34 chicks in each pen of females at the beginning of the experiment.
[c] This pen was discontinued at the end of the thirty-eighth week because of excessive mortality.
[d] This pen was discontinued at the end of the thirty-sixth week because the egg production had increased to such an extent that as far as the relation between feed consumption and growth was concerned, the data were of questionable value.
[e] This pen was discontinued at the end of the thirty-seventh week because of excessive mortality.

FITTING THE EQUATION OF THE CURVE OF DIMINISHING INCREMENT TO THE EXPERIMENTAL DATA

The equation of the curve of diminishing increment was fitted to the feed-consumption and live-weight data obtained from each of the 14 pens. This equation may be written in two ways, of which one is

$$W = A - BR^F \qquad (1)$$

in which, according to Spillman's hypothesis (4),

W = the live weight for any corresponding feed consumption, F;
A = the maximum live weight attainable;
B = the difference between A and the initial live weight, i.e., the total gain in live weight possible;
R = the Spillman ratio, which is the inverse ratio of the gains in live weight resulting from any two successive units of feed consumed (thus, if one unit of feed produces a gain of 0.30 kilogram and the next a gain of 0.27 kilogram,

$$R = \frac{1}{\dfrac{0.30}{0.27}} = \frac{0.27}{0.30} = 0.9), \text{ and}$$

$F =$ the cumulative feed consumption.

Another form in which this equation may be written is

$$W = A - B\epsilon^{-kF} \tag{2}$$

in which W, A, B, and F have the same significance as before, ϵ is the base of the natural system of logarithms, and k is a constant which is related to R by the following equation:

$$R = \epsilon^{-k} \tag{3}$$

The first step in fitting this equation to the data for each pen was to determine the approximate values of the parameters A, B, and k by the rapid method recently described by Hendricks (*1*). After having obtained the approximate values of A, B, and k, the writers employed the following adjustment equation to determine the corrections to be made to the approximate values of the parameters:

$$\frac{\partial f}{W_o \partial A_o} \alpha + \frac{\partial f}{W_o \partial B_o} \beta + \frac{\partial f}{W_o \partial k_o} \kappa = \frac{W - W_o}{W_o} \tag{4}$$

in which

$$\frac{\partial f}{\partial A_o} = 1, \quad \frac{\partial f}{\partial B_o} = -\epsilon^{-k_o F}; \text{ and } \frac{\partial f}{\partial k_o} = FB_c \epsilon^{-k_o F}$$

and α, β, and κ are corrections to be made to A_o, B_o, and k_o, respectively, which are approximations, previously obtained, of the constants A, B, and k; and W and W_o are, respectively, the corresponding observed and calculated live weights.

The corrected values of A, B, and k were readjusted until the corrections became negligible. In most cases only a single adjustment of the parameters was required because the rapid method gave values of the parameters which required only small corrections. The values of R were then calculated by means of the relationship between k and R, i.e., $R = \epsilon^{-k}$. The unit of feed weight, as well as the unit of live weight, used in this study is the kilogram. In applying the various equations, all weights should be expressed in kilograms.

The writers consider the adjustment equation used in this paper, i.e., equation 4, to be superior to the one previously used by Jull and Titus (*3*), for when equation 4 is used, the sum of the squares of the relative residuals is reduced to a minimum, whereas when the other equation is used, the sum of the squares of the absolute residuals is reduced to a minimum.

THE PARAMETERS AND DERIVED CONSTANTS

In order to summarize as briefly as possible the chief numerical results of fitting the equation of the curve of diminishing increment to the data on feed consumption and live weight, the parameters of this equation, as well as several derived constants, have been tabulated in table 3.

TABLE 3.—*Values of the constants of the live weight-feed consumption equations (curve of diminishing increment) and the coefficients of deviation of the observed from the calculated live weights for each of the 14 pens of chicks*

Pen no.	Relative level of feed intake [a]	A	Probable error of A	B	Probable error of B	k	Probable error of k	R [b] (or ε^-k)	kB [c]	Probable error of kB	Coefficient of deviation [d]
	Percent	Kilograms	Kilogram	Kilograms	Kilogram						Percent
MALES											
84	100.0	3.677	±0.031	3.442	±0.030	0.0925	±0.0015	0.9116	0.337	±0.003	4.52
86	68.6	2.956	±.010	2.923	±.010	.1203	±.0007	.8867	.351	±.001	1.56
88	58.0	2.621	±.013	2.588	±.013	.1387	±.0012	.8705	.359	±.002	2.45
90	49.0	2.235	±.010	2.202	±.010	.1627	±.0013	.8499	.358	±.002	1.99
92	39.2	1.654	±.012	1.620	±.012	.2011	±.0024	.8154	.331	±.002	3.00
94	29.4	.985	±.009	.951	±.009	.3719	±.0074	.6894	.354	±.005	5.24
96	19.6	.638	±.009	.606	±.009	.5159	±.0141	.5970	.312	±.005	4.62
FEMALES											
85	100.0	2.165	±0.023	2.131	±0.022	0.1598	±0.0036	0.8523	0.341	±0.005	4.40
87	85.4	2.362	±.027	2.330	±.027	.1489	±.0031	.8617	.347	±.004	3.72
89	73.5	2.163	±.026	2.132	±.026	.1681	±.0033	.8452	.358	±.003	3.53
91	60.9	1.974	±.009	1.941	±.009	.1800	±.0015	.8353	.349	±.002	2.15
93	49.2	1.641	±.006	1.608	±.006	.2190	±.0014	.8033	.352	±.001	1.64
95	36.8	1.233	±.010	1.201	±.009	.2804	±.0033	.7555	.337	±.002	2.99
97	24.5	.645	±.008	.613	±.008	.4778	±.0095	.6201	.263	±.003	3.22
General mean [c] of kB for the 14 pens									.347	±.001	

[a] The level of feed intake is expressed as the percentage of the ad libitum feed consumption.

[b] The numerical value of ϵ^{-k} is equal to the inverse ratio of the gains in live weight resulting from any 2 successive units of feed consumed. This ratio is referred to as the Spillman ratio. Since $R=\epsilon^{-k}$, the probable error of k is also the probable error of $\ln 1/R$.

[c] The numerical value of kB is equal to the maximum efficiency of the feed in producing gains in live weight. It is the value of the left-hand member of $dW/dF=kB^{-kF}$ when $F=0$.

[d] The coefficient of deviation of the observed live weights from the calculated live weights. The coefficient of deviation is analogous to the familiar coefficient of variation. In this case it was calculated by means of the formula

$$\text{C. of D.} = \sqrt{\frac{\Sigma\left[\frac{100(x-x_0)}{x_0}\right]^2}{N-3}},$$

in which x represents an observed value and x_c represents the corresponding calculated value. To show the analogy between the 2 a comparison may be made of the formula for the coefficient of deviation in the case of a 1-constant equation with that for the familiar coefficient of variation. The formula of the former is

$$C. \text{ of } D. = \sqrt{\Sigma \left[\frac{\frac{100(x - x_c)}{x_c}}{N-1} \right]^2},$$

and the formula of the latter may be written

$$C. \text{ of } V. = \sqrt{\Sigma \left[\frac{\frac{100(x - \bar{x})}{\bar{x}}}{N-1} \right]^2}, \text{ since } C. \text{ of } V. = \frac{100\sigma}{\bar{x}} \text{ and } \sigma = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N-1}}.$$

e Weighted on the basis of the probable errors.
f If the values of the maximum efficiency of the feed in the case of pens 96 and 97 are omitted, the general mean maximum efficiency of the feed becomes 0.350 ± 0.001.

INTERPRETATION AND DISCUSSION OF THE EXPERIMENTAL RESULTS

RELATIONSHIP BETWEEN FEED CONSUMPTION AND LIVE WEIGHT AS EXPRESSED BY EQUATION OF CURVE OF DIMINISHING INCREMENT

A comparison of the observed live weights with those calculated by means of the equation of the curve of diminishing increment showed that this equation describes the relationship between feed consumption and live weight with a high degree of accuracy.

To illustrate graphically the excellent agreement between the observed and calculated live weights, the observed average live weights
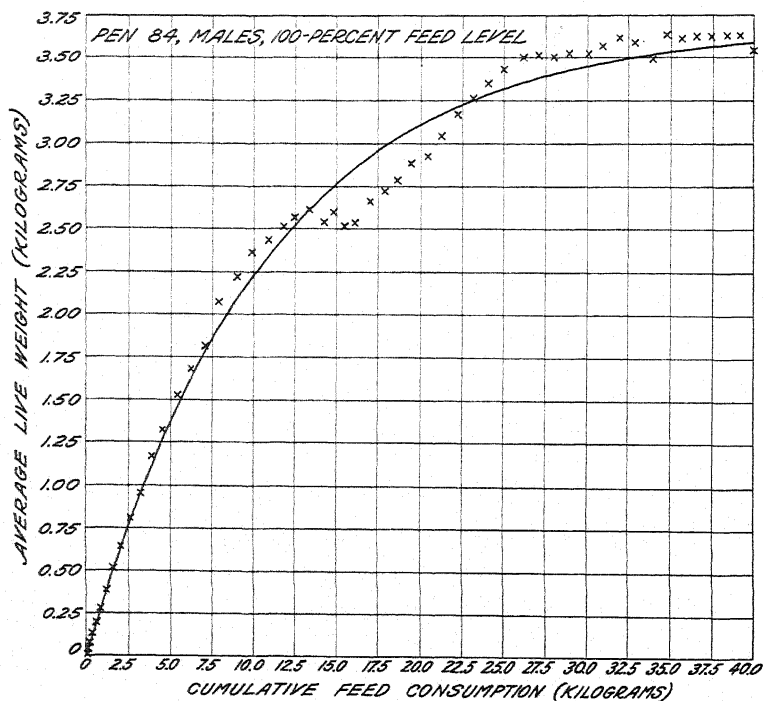
FIGURE 1.—A representative example of one of the poorer fits of the equation of the curve of diminishing increment to the experimental data. The smooth curve was plotted by means of the equation

$$W = 3.67695 - 3.64207 e^{-0.0925462F} \text{ (kg)}.$$

The crosses represent observed average live weights plotted against cumulative feed consumption.

of the cockerels in pens 84 and 86 are plotted, together with the fitted curves, in figures 1 and 2, respectively. Pen 84 was selected as being representative of one of the poorer fits of the equation to the experimental data, whereas pen 86 was selected as being representative of one of the better fits. In the case of pen 84 some of the deviations of the observed live weights from the curve are rather large, but in no instance do they exceed 12 percent of the corresponding calculated values, and the coefficient of deviation is only ±4.52 percent. In the case of pen 86 the largest deviation is less than 7 percent, and the coefficient of deviation has the very low value of ±1.56 percent.

The coefficients of deviation of the observed from the calculated live weights for all 14 pens are given in table 3. The low values of these coefficients are further evidence of the close agreement between the observed and the calculated live weights, and hence of the ability of the equation of the curve of diminishing increment to express accurately the relationship between live weight and feed consumption.

It was of considerable interest to note that for those pens in which egg production did not complicate the picture most of the deviations, when expressed in percentage, were rather small, except in the case of the 2 pens in which the chicks were allowed to eat all they wanted and the 4 pens on the 2 lowest levels of feed intake. This at least indicates the practicability, as well as the desirability, of controlling, according to a predetermined schedule, the feed intake of animals in comparative feeding experiments, so that the feed consumed will be somewhat less than they would eat of their own free will but at least 50 percent of the quantity that they would normally be expected to eat. A level of feed intake equal to approximately 70 percent of the ad libitum level is recommended because in most cases the animals receiving feed at this level of intake may be expected to eat all that is fed them unless the feed is unpalatable, and because, as is shown later, the value of the Spillman ratio for this level is not so greatly different from its value for the ad.libitum level, whereas below the 70-percent level the value of the Spillman ratio decreases with increasing rapidity as the level of feed intake is decreased.

FIGURE 2.—A representative example of one of the better fits of the equation of the curve of diminishing increment to the experimental data. The smooth curve was plotted by means of the equation

$$W = 2.95629 - 2.92281\epsilon^{-0.1202525F} \text{ (kg)}.$$

The crosses represent observed average live weights plotted against cumulative feed consumption.

Titus and Hendricks (*9*) have recorded the observation that when chicks are fed at different levels of intake varying from about 40 percent of ad libitum to ad libitum consumption, the live weights of less than approximately 500 grams may be expressed by a single equation relating live weight to feed consumption, regardless of the level of intake. At first the reason for this phenomenon was not clearly evident, but the present study indicates that this follows as a result of one of the properties of the equation of the curve of diminishing increment. Figures 3 and 4 illustrate this point. The curves of the fitted equations are plotted in figure 3 for males and in figure 4

for females.　An examination of these curves shows that those for the levels of feed intake above 48 percent of the ad libitum feed consumption almost coincide, in the case of both the males and the females, un-
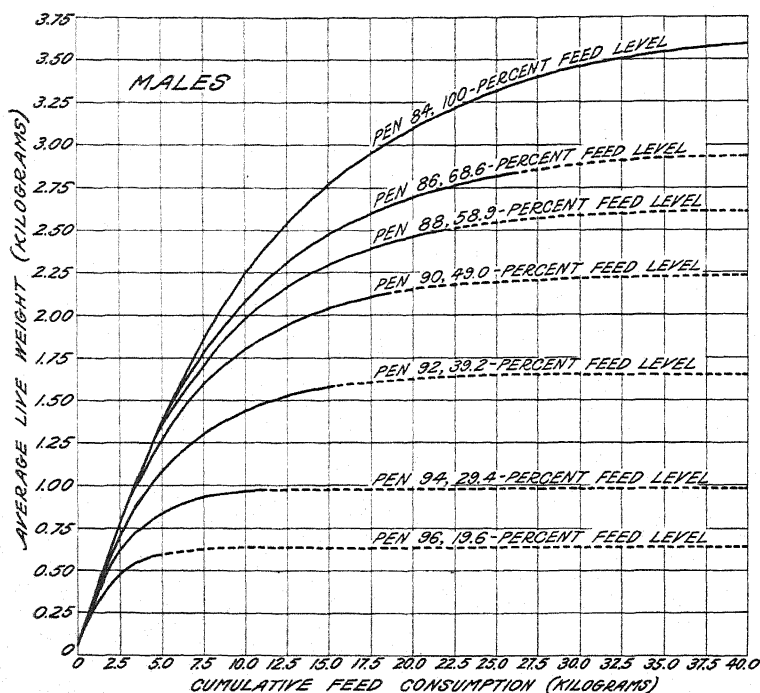
FIGURE 3.—Curves of the fitted equations for the males.　Solid lines represent the growth interval studied; broken lines are extrapolations.
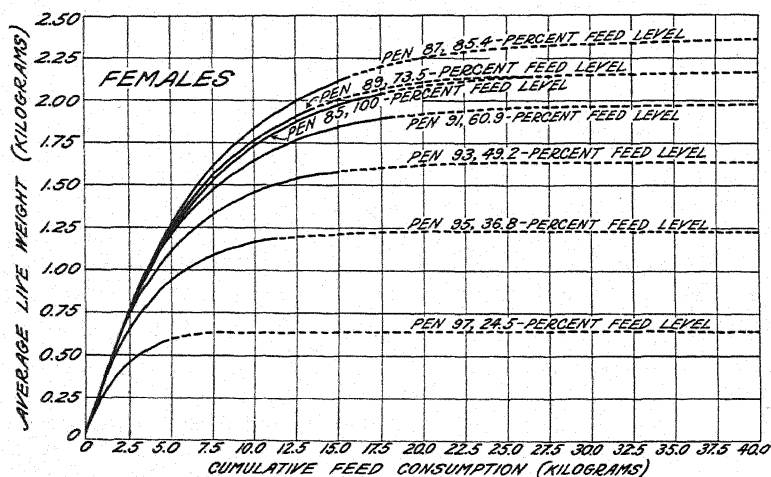
FIGURE 4.—Curves of the fitted equations for the females.　Solid lines represent the growth interval studied; broken lines are extrapolations.

til a live weight between 750 and 1,000 grams is reached; and for levels of feed intake above 58 percent, the curves almost coincide until a live weight of nearly 1,250 grams is reached.

Having observed the close dependence of live weight on feed consumption, one may now attempt to determine the cause of the irregularities in the curves showing the relation between feed consumption and live weight (fig. 1) and between age and live weight (fig. 5) for pen 84 and between age and live weight (fig. 6) for pen 85. In figures 7 and 8 the rate of feed consumption of the males and females, respectively, is plotted against age. Inspection of these figures shows that after about the twelfth week the rate of feed consumption by the chicks in pens 84 and 85 was very irregular. And, in general, it is found that there is a direct relation between the irregularities in the live-weight curves and

FIGURE 5.—Live weights of the males plotted as a function of age. Solid lines represent observed weights; long-dash line for pen 84 was obtained by plotting, against age, the average live weights calculated by means of the equation $W = 3.67695 - 3.64207e^{-0.0925462x}$ (kg); short-dash line for pen 84 represents the weights which would have resulted if the feed consumption had followed the curve, shown in figure 7, representing the approximation of the idealized ad libitum rate of feed consumption.

those in the curves depicting the rate of feed consumption. For
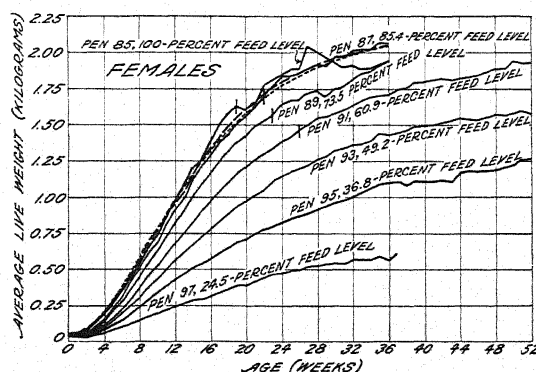
FIGURE 6.—Live weights of the females plotted as a function of age. Solid lines represent observed weights; long-dash line for pen 85 was obtained by plotting, against age, the average live weights of the chicks calculated by means of the equation $W = 2.16495 - 2.13054e^{-0.1595439x}$ (kg); short-dash line represents the weights which would have resulted if the feed consumption of the chicks in pen 85 had followed the approximation of the idealized ad libitum rate of feed consumption curve shown in figure 8. The short lines transecting the curves indicate the approximate age of the females, at beginning of laying, in those pens the feed-consumption data of which were corrected for egg production.

the sake of comparison, approximations of the curves representing idealized ad libitum rate of feed consumption for males and females are shown (by broken lines) in figures 7 and 8, respectively. When the curves representing the observed rate of feed consumption are compared with the approximations of the idealized curves, the irregularities in the observed rate of feed consumption are brought out in a striking manner.

From the explanation just given of the irregularities observed in the curves showing the relation between feed

consumption and live weight, and age and live weight, it follows that in conducting a comparative feeding experiment better results are obtained if, in addition to giving all the groups of chickens the same quantity of feed per head, it is arranged that the rate of feed consumption be a fixed percentage of a carefully made approximation of the idealized rate. Obviously, uniformly regular growth can take place only when the rate of feed consumption closely follows a uniformly regular course.
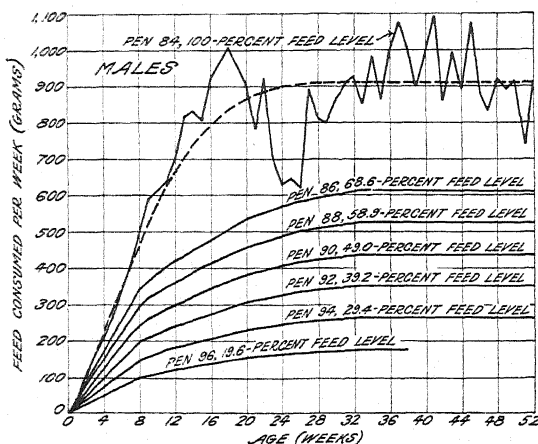
FIGURE 7.—Grams of feed consumed per chick per week in the seven pens of males plotted against age. The solid lines represent the observed feed consumption per chick per week; the broken line is an approximation of the curve showing the idealized ad libitum rate of feed consumption of the males in pen 84.

EFFECT OF THE LEVEL OF FEED INTAKE ON THE NUMERICAL VALUE OF THE SPILLMAN RATIO

According to the data presented in table 3, the Spillman ratio (R in equations 1 and 3) decreases in magnitude as the relative level of feed intake is decreased. It now remains to determine the nature of the relationship between the relative level of feed intake and the numerical value of R. If these two variables are plotted against each other, as in figure 9, it at first appears that the relationship is expressible by the equation of the curve of diminishing increment. However, if a calculation is made of the values of R when the unit of feed consumption is 10 kilograms instead of 1 kilogram, and these new values are plotted against the relative levels of feed intake, the sigmoid shape of the curve at once becomes apparent.

Because of the sigmoid shape of the curve (under the conditions just stated) it was decided to fit the equation

FIGURE 8.—Grams of feed consumed per chick per week in the seven pens of females plotted against age. The solid lines represent the observed feed consumption per chick per week; the broken line is an approximation of the curve showing the idealized ad libitum rate of feed consumption of the females in pen 85. The short lines transecting the curves indicate the approximate age of the females, at beginning of laying, in those pens the feed-consumption data of which were corrected for egg production.

$$ln\frac{y}{a-y} = k(x-b) \tag{5}$$

to the data. When this was done it was found that an excellent fit was obtained, since in no case did the observed values differ from the calculated values by more than 3 percent and the coefficient of deviation was only ±2.13 percent in the case of the males and ±0.95 percent in the case of the females. The results obtained by fitting equation 5 to the two sets of data are given in table 4.

TABLE 4.—*Effect of the level of feed intake on the numerical value of the Spillman ratio, R*

	Males					Females			
Relative level of feed intake [a] (percent)	Spillman ratio, R (or ϵ^{-k})		Difference between observed and calculated		Relative level of feed intake [a] (percent)	Spillman ratio, R (or ϵ^{-k})		Difference between observed and calculated	
	Observed	Calculated [b]	Absolute	Relative		Observed	Calculated [c]	Absolute	Relative
				Percent					*Percent*
100.0	0.9116	0.9096	+0.0020	+0.22	100.0	0.8523	0.8563	−0.0040	−0.47
68.6	.8867	.8928	−.0061	−.68	85.4	.8617	.8543	+.0074	+.87
58.9	.8705	.8763	−.0058	−.66	73.5	.8452	.8499	−.0047	−.55
49.0	.8499	.8458	+.0041	+.49	60.9	.8353	.8380	−.0027	−.32
39.2	.8154	.7937	+.0217	+2.74	49.2	.8033	.8114	−.0081	−1.00
29.4	.6894	.7103	−.0209	−2.94	36.3	.7555	.7476	+.0079	+1.06
19.6	.5970	.5919	+.0051	+.86	24.5	.6201	.6222	−.0021	−.34
Coefficient of deviation.				±2.13	Coefficient of deviation.				±.95

[a] Expressed as percentage of the ad libitum feed consumption.

[b] Calculated by means of the equation, $ln\dfrac{y}{0.91212-y}=0.06568(x-10.2403)$, in which $y=$the Spillman ratio, R, and $x=$the level of feed intake.

[c] Calculated by means of the equation, $ln\dfrac{y}{0.85728-y}=0.07689(x-11.8420)$, in which y and x have the same significance as in the preceding footnote.

Figure 9 clearly shows the relationship between the Spillman ratio and the relative level of feed intake. Although, as previously stated, this ratio decreases as the level of feed intake is decreased, the rate of decrease is relatively slow until a level of about 50 percent is reached, after which it becomes very rapid. This was one of the reasons that earlier in this paper a level of feed intake equal to 70 percent of the ad libitum level was recommended for use in comparative feeding experiments.

By means of the equations given in the footnotes to table 4 one may calculate the value of R with a high degree of accuracy for any relative level of feed intake, at least

FIGURE 9.—Effect of level of feed intake on the Spillman ratio, R (or ϵ^{-k}). The solid-line curves were plotted by means of the equations given in footnotes b and c of table 4.

over the interval studied, i.e., between approximately 20 and 100 percent of the ad libitum feed consumption. In the case of the males it

is found that R is equal to 0.9096 for the ad libitum level and 0.8945 for the 70-percent level; and in the case of the females the corresponding values of R are 0.8563 and 0.8476, respectively.

With the exception of swine, the growing animal makes larger gains in live weight when full-fed than when underfed, and makes them more economically. Hence, two very practical questions arise:(1) What constitutes underfeeding? and (2) how serious is a given degree of underfeeding? The answer to the first question is that anything materially less than the ad libitum level of feed intake is underfeeding in the case of the growing animal. The second question can be answered satisfactorily only when the case is accurately specified. Nevertheless it may be shown, by means of the figures given in the preceding paragraph, that the first 4 kilograms of feed consumed by a growing cockerel, if fed at the 70-percent level, are utilized nearly 97 percent[4] as efficiently as they would be if fed at the 100-percent, or ad libitum, level. In the case of the growing pullet the feed is utilized slightly more than 98 percent as well at the 70-percent level as it is at the 100-percent level.

When an animal is grossly underfed, the situation is quite different. For example, a pullet utilizes her first 4 kilograms of feed only about 83 percent as well at the 40-percent level of intake as at the 100-percent level, and the cockerel utilizes his feed only about 79 percent as well.

EFFECT OF THE LEVEL OF FEED INTAKE ON THE TOTAL GAIN POSSIBLE

The data presented in table 5 show that B, the total gain possible on any given level of feed intake, decreases as the level of feed intake is decreased. This is shown graphically in figure 10, which indicates the mathematical form of the relationship between the two. Too few data are available to enable one to determine the precise form of the equation most suitable for expressing B as a function of level of feed intake; however, to enable one to estimate the numerical value of B for any relative level of feed intake within the range studied, the data were graduated by means of the equation

$$y = a - b\epsilon^{-kx} \tag{6}$$

In table 5 the numerical values of B, calculated by means of this equation, are compared with the observed values. Although the

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[4] For computing the relative efficiency of utilization of the feed when fed at two different levels, a function involving only R is to be preferred to one involving both B and R, since R (table 4) was graduated with much greater precision than B (table 5). A suitable function involving only R may be obtained as follows: Since $W = A - BR^F$, the initial weight, W_1, is given by the equation $W_1 = A - B$. It follows, then, that the gain, G, may be expressed as follows: $G = W - W_1 = (A - BR^F) - (A - B) = B(1 - R^F)$. Hence, the ratio of the gain resulting from the first 4 kilograms of feed at the 70-percent level of intake to the gain resulting from the same quantity of feed at the ad libitum level is

$$\frac{G_{70}}{G_{100}} = \frac{B_{70}\ (1 - R^4_{70})}{B_{100}(1 - R^4_{100})}.$$

Since the product of k and B is a constant, $k_{70}B_{70} =$ a constant $= k_{100}B_{100}$; hence

$$\frac{B_{70}}{B_{100}} = \frac{k_{100}}{k_{70}}.$$

Also, since $\epsilon^{-k} = R$, $k = -2.3026 \log R$. Hence, one may write

$$\frac{G_{70}}{G_{100}} = \frac{B_{70}(1 - R^4_{70})}{B_{100}(1 - R^4_{100})} = \frac{k_{100}(1 - R^4_{70})}{k_{70}(1 - R^4_{100})} = \frac{(-2.3026 \log R_{100})\ (1 - R^4_{70})}{(-2.3026 \log R_{70})\ (1 - R^4_{100})} = \frac{(-\log R_{100})\ (1 - R^4_{70})}{(-\log R_{70})\ (1 - R^4_{100})} =$$

$$\frac{(-\log 0.9096)\ (1 - \overline{0.8945})}{(-\log 0.8945)\ (1 - \overline{0.9094})} = \frac{(0.0411495)\ (0.3598)}{(0.0484197)\ (0.3155)} = \frac{0.01480559010}{0.01527641535} = 0.9692, \text{ or approximately 97 percent.}$$

agreement between the two is not so good as is to be desired, especially in the case of the males, one can interpolate by means of equation 6 the expected total gain for any level of feed intake with a fair degree of accuracy.

Table 5 shows that the calculated value of B for the pen of males and the pen of females which ate all the feed they wanted is appreciably greater than the observed value in both cases. This, no doubt, is due in part to the fact that the chicks in these two pens wasted some of the feed given them. The value of B for the pen of males on the 29.4-percent level of feed intake is out of line with the values of this parameter for the

FIGURE 10.—Effect of level of feed intake on B, the total gain possible. The dash-line curves were plotted by means of the equations given in footnotes b and c of table 5.

other pens of males; likewise the value of A, the maximum average live weight attainable, is out of line. The reason for these apparent discrepancies is not known.

TABLE 5.—*Effect of level of feed intake on numerical value of B, total gain possible*

	Males					Females			
Relative level of feed intake [a] (percent)	B (total gain possible)		Difference between observed and calculated		Relative level of feed intake [a] (percent)	B (total gain possible)		Difference between observed and calculated	
	Observed	Calculated [b]	Absolute	Relative		Observed	Calculated [c]	Absolute	Relative
	Kilograms	*Kilograms*	*Kilogram*	*Percent*		*Kilograms*	*Kilograms*	*Kilogram*	*Percent*
100.0__	3.642	3.845	−0.203	−5.28	100.0__	[d] 2.131	2.459	−0.328	[e] −13.34
68.6__	2.923	2.837	+.086	+3.02	85.4__	2.330	2.317	+.013	+.58
58.9__	2.588	2.465	+.123	+5.00	73.5__	2.132	2.154	−.022	−1.04
49.0__	2.202	2.050	+.152	+7.42	60.9__	1.941	1.919	+.022	+1.12
39.2__	1.620	1.602	+.018	+1.14	49.2__	1.608	1.623	−.015	−.89
29.4__	.951	1.113	−.162	−14.54	36.8__	1.201	1.195	+.006	+.54
19.6__	.606	.579	+.027	+4.64	24.5__	.613	.613	_____	
Coefficient of deviation___	_____	_____	_____	±9.37	Coefficient of deviation___	_____	_____	_____	[f] ±6.74

[a] Expressed as percentage of ad libitum feed consumption.
[b] Calculated by means of the equation, $y=6.97497−7.61302e^{−0.0088843x}$, in which $y=B$, the total gain possible, and $x=$ the level of feed intake.
[c] Calculated by means of the equation, $y=2.77507−4.03429e^{−0.0254642x}$, in which y and x have the same significance as in the preceding footnote.
[d] This value of B is obviously out of line with the other values of B and, hence, was not used in fitting the equation, $y=a−be^{−kx}$, to the data for the females.
[e] When this value is omitted (for the reason stated in footnote [d]) the coefficient of deviation becomes ±1.12 percent.
[f] As stated in footnote [e], when the first value in this column is omitted, the coefficient of deviation is reduced from 6.74 to 1.12 percent.

SIGNIFICANCE OF PARAMETERS OF EQUATION OF CURVE OF DIMINISHING INCREMENT WHEN APPLIED TO DATA ON FEED CONSUMPTION AND LIVE WEIGHT

Jull and Titus (*3*), in their earlier study of the growth of chickens in relation to feed consumption, were led to the conclusion that the

parameters A and B (M and A, respectively, in their paper) could be considered only as empirical constants. In the present study a much more extensive set of data was obtained, and in the interval between the two investigations a very satisfactory and suitable method of fitting the equation was developed.

A comparison of the observed and calculated live weights showed that the average initial live weights, i.e., the average live weights before any feed was consumed, were reproduced with a high degree of precision by the equation of the curve of diminishing increment. Furthermore, this comparison showed that, on the whole, the observed and calculated average live weights agreed extremely well throughout the growth intervals studied, especially in those pens in which the feed consumption was controlled and in which the level of feed intake was more than 50 percent of the ad libitum level. These considerations lead the writers to the following conclusions regarding the significance of the three parameters of the equation of the curve of diminishing increment.

(1) A represents the maximum average live weight attainable on a given level of feed intake, provided that that level is maintained and that no appreciable fattening occurs. In the case of chickens allowed to eat all they want of an adequate diet, A represents the maximum average live weight attainable, provided no appreciable fattening occurs.

(2) B represents the difference between the maximum average live weight, A, and the average initial live weight; that is, it is numerically equivalent to the average total gain which can be made, if no appreciable fattening occurs.

(3) R, the Spillman ratio, is the inverse ratio of the gains in live weight resulting from any two successive units of feed consumed.

Since the first derivative of the equation of the curve of diminishing increment may be written

$$dW/dF = kB\epsilon^{-kF} \qquad (6)$$

or

$$dW/dF = kBR^F \qquad (6')$$

it follows that kB is equal to the maximum efficiency of the feed in producing gains in live weight, if this efficiency is defined as the ratio of the gain in live weight to the quantity of feed required to produce the gain. The efficiency of successive units of feed decreases in geometrical progression and the magnitude of the decrease is determined by the Spillman ratio, R (or ϵ^{-k}).

In regard to its significance, kB should not be confused with kA, which the writers proposed in an earlier paper (2) as a measure of feed efficiency. The former represents the maximum efficiency which a feed may actually have and the latter represents the maximum efficiency which it would have if the animal to which it is fed had an inital live weight of zero. The value of kA is dependent on the maximum live weight attainable by the animal, whereas the value of kB is dependent on both the maximum and initial live weights, since

$$B = A - w_1 \text{ (w_1 being the initial live weight)}.$$

The values of the maximum efficiency of the feed (kB) are given in table 3 for each of the 14 pens. Although there appears to be a slight tendency for the maximum efficiency to increase at first and then to decrease as the level of feed intake is decreased, there is a marked agreement among the values. If the values for the two pens on the lowest absolute level of feed intake are eliminated, the weighted mean value of kB is 0.350 ± 0.001 and the range is from 0.331 to 0.359.

The various numerical values of A, B, and R in the equation of the curve of diminishing increment and the parameters of the equations which are given for expressing the relationship between (1) R (the Spillman ratio) and the level of feed intake and (2) B (the total gain possible) and the level of feed intake hold only for the particular diet fed and for the particular crossbreed of chicken used. However, it is reasonably certain that, with suitable values for the parameters, the several equations used in this study will hold for any adequate diet and for any breed of chickens.

EFFECT OF SEX ON UTILIZATION OF FEED

If, for corresponding absolute levels of feed intake, one compares the numerical values of the Spillman ratio for the two sexes, he finds that for the first three of the levels less than the ad libitum level the values of R are greater for the males than they are for the females, but on the two lowest absolute levels the opposite is true. If it is assumed that the product kB is constant regardless of sex or level of feed intake (and the values of this product given in table 3 seem to indicate that this may be the case), one is led to the conclusion that on the higher absolute levels of feed intake the males of this crossbreed are more efficient in the utilization of feed than the females, but on the lower levels the latter are the more efficient.

Even if one does not make this assumption regarding the constancy of the product kB, the conclusion still appears to hold for the following reasons:

(1) The efficiency of the feed is given by the equation

$$\text{Efficiency} = dW/dF = kBR^F;$$

(2) The Spillman ratio, R, decreases at a greater rate than does the product kB after the latter's apparent maximum is reached;

(3) The values of the Spillman ratio on the higher levels of feed intake are greater for the males than for the females; and

(4) On the two lowest levels the opposite of the preceding statement is true.

If, instead of comparing the efficiency of the males and females on the basis of the absolute levels of feed intake, one makes the comparison on the basis of the relative levels of feed intake, it is found that the males are the more efficient for all the relative levels studied.

SUMMARY AND CONCLUSIONS

Extensive and critical data were obtained on the relationship between feed consumption and live weight in the case of crossbred chickens (Rhode Island Red males mated to Barred Plymouth Rock females). Seven pens of males and seven pens of females were fed

at different levels of feed intake, including the ad libitum and six lower levels, for a period of 52 weeks. The weight of feed consumed per chicken per week and the average live weight of the chickens at the end of each week were obtained.

By means of a suitable method, the equation of the curve of diminishing increment was fitted to the data recorded for each of the 14 pens of chickens. It was found that the average live weights computed by means of this equation agreed very closely with the observed average live weights. It was also found that a rational meaning could be assigned to the three parameters of this equation.

It was possible to describe accurately, by means of a simple equation, the relationship between the level of feed intake and the Spillman ratio. It is recommended that in comparative feeding experiments all the groups be fed at a level of feed intake equal to 70 percent of an approximation of the idealized ad libitum feed consumption. At this level the value of the Spillman ratio is nearly as large as it is at the ad libitum level. Under these conditions the several groups would eat the same quantity of feed, unless it were unpalatable, and the feed consumption would follow a definite course. The latter point is of importance since uniformly regular growth can take place only when the feed consumption follows a uniformly regular course.

The following conclusions are drawn:

The equation of the curve of diminishing increment, $W = A - BR^F$, describes with a high degree of accuracy the relationship between feed consumption and live weight over a wide range of levels of feed intake.

A in the above equation represents the maximum live weight attainable on a given level of feed intake, provided this level is maintained and no appreciable fattening occurs.

B in the above equation represents the maximum gain possible under the conditions just stated.

R, the Spillman ratio, is the inverse ratio of the gains in live weight resulting from any two successive units of feed consumed.

The relationship between level of feed intake and the numerical value of R is expressible, over the range of levels studied, by the equation,

$$ln \frac{y}{a-y} = k(x-b), \text{ in which}$$

$y =$ the Spillman ratio, $x =$ the relative level of feed intake, and a, k, and b are constants.

The relationship between level of feed intake and the numerical value of B is expressible, for the range of levels studied, with a fair degree of accuracy by the equation

$$y = a - b\epsilon^{-kx}, \text{ in which}$$

$y = B$, the total gain possible; $x =$ the relative level of feed intake, and a, b, and k are constants.

On the higher absolute levels of feed intake the males of the crossbreed studied are more efficient than the females in their utilization of feed for growth, whereas on the very low absolute levels the opposite is true.

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COMPARISON OF THE PULLORIN AND THE RAPID WHOLE-BLOOD AGGLUTINATION TESTS FOR PULLORUM DISEASE [1]

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INTRODUCTION

The control of pullorum disease is based on the diagnosis of the carrier fowl and its elimination from breeding. Various tests are at present in use in different parts of the country for diagnosing the pullorum-disease carrier. The "long" or "tube" agglutination test has been in use since 1913 and has been developed to a considerable degree of refinement. The principal objections to it are its slowness and the comparatively great expense and labor involved in its application. To meet these objections more simple methods have since been devised. Some of these retain the principle of serum-antigen agglutination, but one is based upon the allergic reaction of living tissue. This last is known as "the pullorin test" or "the intradermic test" for pullorum disease. The name "pullorin" refers to the reagent employed.

However, in the 17 years in which it has been known, the pullorin test has not been widely accepted by poultry-disease diagnosticians, many of whom have been skeptical as to its accuracy. The experiments recorded in this paper were conducted with a view to ascertaining more fully the relative diagnostic value of the pullorin test as compared with the rapid whole-blood agglutination test [3] for pullorum disease. The latter test involves the use of a stained antigen. The rapid whole-blood agglutination test was selected for the comparisons because it had the advantages claimed for the pullorin test with regard to simplicity and economy. The experiments also included some corroborative tests by the tube agglutination method.

HISTORICAL REVIEW

Ward and Gallagher [4] in 1917 first reported the development of an intradermic test for pullorum disease. These workers prepared an allergic reagent by inoculating broth with cultures of *Salmonella pullorum*, incubating at 37° C. for 1 month, holding the broth in an ice box for about 6 months, then passing it through a Berkefeld filter, and finally preserving it with 0.5-percent phenol. This product was

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injected intradermically into one of the wattles of the fowl to be tested. After 24 hours the injected wattles of infected fowls were swollen, and those of uninfected fowls were not swollen. The swelling of the wattles of reactors increased noticeably in the next 24 hours.

These authors later modified their product by growing the organism in broth for 1 month at 37.5° C., and killing it by heating at 60° for 1 hour in a water bath. They then carbolized it to 0.5 percent. This product was employed without filtration.

The formula thus developed has been largely the basis of most of the commercial and experimental pullorins since used. Pullorins of the type of that first described are usually referred to as noncellular, or cell-free pullorins, whereas those of the second type are known as cellular pullorins.

A number of contemporary workers have investigated the possibilities of the diagnosis of pullorum disease by means of an allergic test. Rettger and Plastridge [5] state: "The beliefs expressed at the 1930 Poultry Congress in London were, with one exception, distinctly unfavorable to the pullorin test." A translation of Rosell's [6] comments on the comparative results between the pullorin test and the stained-antigen, rapid whole-blood test is as follows:

In a comparative test of the efficiency of this method with those employing different kinds of pullorin, one of which was of the soluble type which I prepared by a new method, the results achieved in collaboration with Drs. Hall and Bunyea were favorable to the whole-blood method which possessed the advantage of requiring only one visit to the poultry plant. This method assuredly provides the more practical means of combating pullorum disease.

EXPERIMENTAL PROCEDURE

The experiments comprised field trials of the pullorin test in comparison with the stained-antigen, rapid whole-blood agglutination test [7] applied to previously untested poultry flocks in the vicinity of Washington, D.C. The plan of this investigation included the use of several commercial brands of pullorin and one or more pullorins prepared by research workers. Arrangements were accordingly made with four nearby flock owners for the application of the comparative tests, which were to be conducted simultaneously. However, neither test was to be set up as the standard by which to judge the merits or defects of the other. The value of a diagnostic test consists in its ability to detect the presence of the elements or processes of disease. Therefore, the criterion proposed in these experiments was that of the laboratory demonstration of *Salmonella pullorum* in the carcasses of reacting fowls selected from the four flocks tested.

In the case of fowls slaughtered from the last three flocks, a tube test was included at autopsy, as having some corroborative value with reference to the rapid whole-blood agglutination test findings.

Five different pullorins were used, two of which were noncellular and three cellular. The two experimental pullorins, R and S, were prepared, respectively, by J. M. Rosell [8] and George W. Stiles. Rosell gives the following information [9] concerning his method of preparation of the cell-free pullorin:

[5] RETTGER, L. F., and PLASTRIDGE, W. H. PULLORUM DISEASE OF DOMESTIC FOWL. A MONOGRAPH. Conn. (Storrs) Agr. Expt. Sta. Bull. 178, pp. [109]–192 illus. 1932.
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[8] ROSELL, J. M. See footnote 6.
[9] ROSELL, J. M. Informal communication.

Five strains of *Salmonella pullorum* (B.A.I. strains 10, 11, 14, 17, and 20) were used in preparing the pullorin. Each strain was grown separately in flasks containing 20 cc of glucose-peptone broth adjusted to a reaction of pH 7.2. The flasks were incubated for 10 days at 37° C. At the end of this period sufficient sterile sodium hydroxide was added so that the broth gave a clear alkaline reaction, by using phenolphthalein as an indicator. It was again incubated for 3 days at 37° or 40° C. in order to obtain a better autolysis of the cells.

After the 3-day incubation period, the culture were frozen for many hours by the use of dry ice, and then melted at 45° C. This procedure of freezing and melting was repeated twice in order that a more complete autolysis be obtained. Microscopically, smears revealed that most of the cells were autolysed.

After purity tests were made the different culture autolysates were mixed and centrifuged in large centrifugal tubes. The supernatant fluid was removed by decantation. To the reunited sediments 20 cc of 0.75-percent sterile potassium hydroxide was added, and this was then shaken and heated at 50° C. until a gelatinous fluid was obtained. This fluid was then centrifuged and the supernatant fluid was added to the supernatant fluid obtained from the first centrifugation. This liquid was adjusted to a pH of 7.2 by adding N/10 hydrochloric acid.

To obtain a more concentrated pullorin the liquid was submitted to a process of evaporation without heating it over 48° C. The liquid was placed in a shallow porcelain pan which was maintained in a water bath of 48° C. for 5 hours. During this time air was allowed to pass through the liquid by means of small glass pipes, and the air currents from an electric fan passed over the surface of the liquid. By this means 1,000 cc of the fluid was evaporated to 666 cc.

Sufficient carbolic acid was then added to the product so that it would contain 0.3 percent of this preservative. It was then filtered through a Mandler candle and transferred aseptically into 20-cc sterile rubber-stoppered vials.

The pullorin supplied by Dr. Stiles was prepared by him essentially after the modified Ward and Gallagher formula, as follows: Five or six flasks of broth were inoculated with separate strains of *Salmonella pullorum*, incubated for at least 1 month, heated at 60° C. for 2 hours, carbolized, and tested for sterility. They were mixed before use.

No information is available as to the methods used in the manufacture of the several commercial pullorins.

The method employed in determining the presence of *Salmonella pullorum* infection in the slaughtered birds was the same as that used by Bunyea and Hall,[10] namely, the aseptic removal and crushing of the entire ovary or testicle, which was then placed in a culture flask of beef infusion broth containing brilliant-green dye in the proportion of 1 to 50,000. Individual colonies of *S. pullorum* were recovered from subcultures made from the brilliant-green broth cultures onto plain agar. The organisms were identified in each instance in the manner described by Bunyea and Hall.

RESULTS OBTAINED

Table 1 shows the percentage of agreements and disagreements between the reactions (1) to the various pullorins used intradermically in the pullorin test and (2) to results obtained with the rapid whole-blood agglutination test.

10 BUNYEA, H., and HALL, W. J. THE RELATION OF AGGLUTINATION REACTION TO SALMONELLA PULLORUM INFECTION IN HENS, AND OBSERVATIONS ON THE DIAGNOSTIC EFFICIENCY OF TEST METHODS. Jour. Amer. Vet. Med. Assoc. (n.s. 33) 80: 491–496, illus. 1932.

injected intradermically into one of the wattles of the fowl to be tested. After 24 hours the injected wattles of infected fowls were swollen, and those of uninfected fowls were not swollen. The swelling of the wattles of reactors increased noticeably in the next 24 hours.

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In a comparative test of the efficiency of this method with those employing different kinds of pullorin, one of which was of the soluble type which I prepared by a new method, the results achieved in collaboration with Drs. Hall and Bunyea were favorable to the whole-blood method which possessed the advantage of requiring only one visit to the poultry plant. This method assuredly provides the more practical means of combating pullorum disease.

EXPERIMENTAL PROCEDURE

The experiments comprised field trials of the pullorin test in comparison with the stained-antigen, rapid whole-blood agglutination test [7] applied to previously untested poultry flocks in the vicinity of Washington, D.C. The plan of this investigation included the use of several commercial brands of pullorin and one or more pullorins prepared by research workers. Arrangements were accordingly made with four nearby flock owners for the application of the comparative tests, which were to be conducted simultaneously. However, neither test was to be set up as the standard by which to judge the merits or defects of the other. The value of a diagnostic test consists in its ability to detect the presence of the elements or processes of disease. Therefore, the criterion proposed in these experiments was that of the laboratory demonstration of *Salmonella pullorum* in the carcasses of reacting fowls selected from the four flocks tested.

In the case of fowls slaughtered from the last three flocks, a tube test was included at autopsy, as having some corroborative value with reference to the rapid whole-blood agglutination test findings.

Five different pullorins were used, two of which were noncellular and three cellular. The two experimental pullorins, R and S, were prepared, respectively, by J. M. Rosell [8] and George W. Stiles. Rosell gives the following information [9] concerning his method of preparation of the cell-free pullorin:

[5] RETTGER, L. F., and PLASTRIDGE, W. H. PULLORUM DISEASE OF DOMESTIC FOWL. A MONOGRAPH. Conn. (Storrs) Agr. Expt. Sta. Bull. 178, pp. [109]–192 illus. 1932.
[6] ROSELL, J. M. PROGRÈS ET NORMES SUR QUELQUES POINTS D'ACTUALITÉ EN PATHOLOGIE ANIMALE. Rev. Inst. Agr. Oka 7 (3): 100–104. 1933.
[7] SCHAFFER, J. M., MACDONALD, A. D., HALL, W. J., and BUNYEA, H. See footnote 3.
[8] ROSELL, J. M. See footnote 6.
[9] ROSELL, J. M. Informal communication.

Five strains of *Salmonella pullorum* (B.A.I. strains 10, 11, 14, 17, and 20) were used in preparing the pullorin. Each strain was grown separately in flasks containing 20 cc of glucose-peptone broth adjusted to a reaction of pH 7.2. The flasks were incubated for 10 days at 37° C. At the end of this period sufficient sterile sodium hydroxide was added so that the broth gave a clear alkaline reaction, by using phenolphthalein as an indicator. It was again incubated for 3 days at 37° or 40° C. in order to obtain a better autolysis of the cells.

After the 3-day incubation period, the culture were frozen for many hours by the use of dry ice, and then melted at 45° C. This procedure of freezing and melting was repeated twice in order that a more complete autolysis be obtained. Microscopically, smears revealed that most of the cells were autolysed.

After purity tests were made the different culture autolysates were mixed and centrifuged in large centrifugal tubes. The supernatant fluid was removed by decantation. To the reunited sediments 20 cc of 0.75-percent sterile potassium hydroxide was added, and this was then shaken and heated at 50° C. until a gelatinous fluid was obtained. This fluid was then centrifuged and the supernatant fluid was added to the supernatant fluid obtained from the first centrifugation. This liquid was adjusted to a pH of 7.2 by adding N/10 hydrochloric acid.

To obtain a more concentrated pullorin the liquid was submitted to a process of evaporation without heating it over 48° C. The liquid was placed in a shallow porcelain pan which was maintained in a water bath of 48° C. for 5 hours. During this time air was allowed to pass through the liquid by means of small glass pipes, and the air currents from an electric fan passed over the surface of the liquid. By this means 1,000 cc of the fluid was evaporated to 666 cc.

Sufficient carbolic acid was then added to the product so that it would contain 0.3 percent of this preservative. It was then filtered through a Mandler candle and transferred aseptically into 20-cc sterile rubber-stoppered vials.

The pullorin supplied by Dr. Stiles was prepared by him essentially after the modified Ward and Gallagher formula, as follows: Five or six flasks of broth were inoculated with separate strains of *Salmonella pullorum*, incubated for at least 1 month, heated at 60° C. for 2 hours, carbolized, and tested for sterility. They were mixed before use.

No information is available as to the methods used in the manufacture of the several commercial pullorins.

The method employed in determining the presence of *Salmonella pullorum* infection in the slaughtered birds was the same as that used by Bunyea and Hall,[10] namely, the aseptic removal and crushing of the entire ovary or testicle, which was then placed in a culture flask of beef infusion broth containing brilliant-green dye in the proportion of 1 to 50,000. Individual colonies of *S. pullorum* were recovered from subcultures made from the brilliant-green broth cultures onto plain agar. The organisms were identified in each instance in the manner described by Bunyea and Hall.

RESULTS OBTAINED

Table 1 shows the percentage of agreements and disagreements between the reactions (1) to the various pullorins used intradermically in the pullorin test and (2) to results obtained with the rapid whole-blood agglutination test.

[10] BUNYEA, H., and HALL, W. J. THE RELATION OF AGGLUTINATION REACTION TO SALMONELLA PULLORUM INFECTION IN HENS, AND OBSERVATIONS ON THE DIAGNOSTIC EFFICIENCY OF TEST METHODS. Jour. Amer. Vet. Med. Assoc. (n.s. 33) 80: 491–496, illus. 1932.

TABLE 1.—*Percentages of agreements and disagreements between results of the pullorin test and the rapid whole-blood agglutination test*

Pullorin used		Birds tested	Agreements [1]		Disagreements [1]	
Source and designation	Type		Pullo-rin +, agglutina-tion +	Pullo-rin −, agglutina-tion −	Pullo-rin +, agglutina-tion −	Pullo-rin −, agglutina-tion +
		Number	*Percent*	*Percent*	*Percent*	*Percent*
Experimental:						
R	Noncellular	218	18.4	49.5	5.5	26.6
S	Cellular	107	23.4	44.9	12.0	19.7
Commercial:						
1	___do__	224	1.3	66.5	3.6	28.6
2	Noncellular	182	17.0	52.2	14.8	16.0
3	Cellular	129	2.3	71.3	6.2	20.2
Total or average		860	11.8	57.2	7.9	23.0

[1] + indicates a positive reaction; −, a negative reaction.

From table 1 it may be observed that the total average agreement in both positive and negative cases is 69 percent, whereas the total average disagreements aggregate 31 percent. Marked fluctuations for the various pullorins are plainly evident.

Tables 2 to 4 show comparisons of the results obtained from the pullorin tests, and the rapid whole-blood agglutination tests, based on their agreement or disagreement with bacteriological findings. Tables 3 and 4 include results obtained from the tube agglutination test, used post mortem.

TABLE 2.—*Comparison of results obtained from pullorins R and S and the rapid whole-blood agglutination test, based on their agreement or disagreement with bacteriological findings*

Fowl no.	Reaction of bird to—			Post-mortem findings			Agreement (+) or disagreement (−) between bacteriological findings and—	
	Pullorin R	Pullorin S	Rapid whole-blood aggluti-nation test	Typical lesions noted	*Salmonella pullorum* isolated		Pullorin test	Rapid whole-blood ag-glutina-tion test
30	Negative		Positive	No	Yes		−	+
414	___do__		Negative	No	No		+	+
13	Positive		___do__	Yes	Yes		+	−
70	___do__		___do__	No	No		−	+
287	___do__		___do__	No	No		−	+
172	___do__		___do__	No	No		−	+
422	___do__		___do__	No	No		−	+
74	Negative		___do__	Yes	Yes		−	+
10	___do__		___do__	Yes	Yes		−	+
90	___do__		___do__	Yes	Yes		−	+
148		Positive	___do__	Suspicious	No		−	+
101		___do__	___do__	___do__	No		−	+
191		___do__	___do__	No	No		−	+
135		___do__	___do__	Suspicious	No		−	+
230		Negative	Positive	Yes	Yes		−	+

TABLE 3.—*Comparison of results obtained from commercial pullorins no. 1, no. 2, and no. 3, and the rapid whole-blood and tube agglutination tests, based on their agreement or disagreement with bacteriological findings*

PULLORIN NO. 1

Fowl no.	Reaction of bird to—		Post-mortem findings					Agreement (+) or disagreement (−) between bacteriological findings and—	
	Pullorin test	Rapid whole-blood agglutination test	Tube agglutination test with blood-serum dilutions of—			Typical lesions noted	*Salmonella pullorum* isolated	Pullorin test	Rapid whole-blood agglutination test
			1:25	1:50	1:100				
95	Negative	Positive	Positive	Positive	Positive	No	Yes	−	+
76	...do	...do	...do	...do	...do	No	No	+	−
83	...do	...do	...do	...do	...do	No	No	+	−
451	...do	...do	...do	...do	...do	Yes	Yes	−	+
107	Positive	Negative	Partial	Partial	Partial	No	No	−	+
57	Negative	Positive	Positive	Positive	Positive	No	No	+	−
25	...do	...do	Slight	Partial	...do	No	Yes	−	+
140	...do	...do	Positive	Positive	Partial	No	No	+	−
82	...do	...do	...do	...do	Positive	No	Yes	−	+

PULLORIN NO. 2

Fowl no.	Pullorin test	Rapid whole-blood agglutination test	1:25	1:50	1:100	Typical lesions noted	*Salmonella pullorum* isolated	Pullorin test	Rapid whole-blood agglutination test
2760	Positive	Positive	Positive	Positive	Partial	No	No	−	−
2766	Negative	...do	...do	Negative	Negative	No	No	+	−
6308	Positive	...do	...do	...do	...do	No	Yes	+	+
9182	...do	...do	...do	Positive	Positive	Yes	Yes	+	+
6332	Negative	...do	...do	...do	Negative	No	Yes	−	+
2756	Positive	...do	...do	...do	Positive	No	No	−	−
2747	Negative	...do	...do	...do	...do	No	Yes	−	+
2730	Positive	...do	...do	...do	Negative	Yes	Yes	+	+
6338	Negative	...do	...do	...do	Positive	Yes	Yes	−	+
6376	Positive	...do	...do	...do	...do	Yes	Yes	+	+
2759	Negative	...do	...do	...do	Slight	No	No	+	−
6386	Positive	...do	...do	...do	Positive	Yes	Yes	+	+

PULLORIN NO. 3

Fowl no.	Pullorin test	Rapid whole-blood agglutination test	1:25	1:50	1:100	Typical lesions noted	*Salmonella pullorum* isolated	Pullorin test	Rapid whole-blood agglutination test
170	Negative	Positive	Positive	Positive	Partial	Yes	Yes	−	+
123	...do	...do	...do	...do	Positive	No	No	+	−
164	...do	...do	...do	...do	...do	Yes	Yes	−	+
177	...do	...do	...do	...do	...do	Yes	Yes	−	+
175	...do	...do	...do	...do	Partial	Yes	Yes	−	+
966	...do	...do	...do	...do	...do	Suspicious	Yes	−	+
993	...do	...do	...do	Partial	Positive	...do	Yes	−	+
181	...do	...do	...do	Positive	...do	Yes	Yes	−	+
967	...do	...do	...do	...do	...do	Yes	Yes	−	+
159	...do	...do	...do	...do	...do	Yes	Yes	−	+
982	...do	...do	...do	...do	...do	No	Yes	−	+
149	...do	...do	...do	...do	Partial	No	Yes	−	+
169[a]	Positive	Negative				No	No	−	+
124	...do	...do	Negative	Negative	Negative	No	No	−	+
171	...do	...do	...do	...do	...do	No	No	−	+
156	...do	...do	...do	...do	...do	No	No	−	+

[a] This bird was found dead; consequently no tube agglutination test was made.

TABLE 4.—*Summary of agreements between the diagnostic test reactions, bacteriological findings, and results of the tube agglutination test used post mortem*

Pullorin used	Fowls examined post mortem	Agreement between pullorin test and—		Agreement between rapid whole-blood agglutination test and—	
		Bacteriological findings	Tube agglutination test	Bacteriological findings	Tube agglutination test
Experimental:	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
R_____	10	20.0	-----------	90.0	-----------
S_____	5	0.0	-----------	100.0	-----------
Commercial:					
1_____	9	45.5	0.0	55.5	100.0
2_____	12	58.3	58.3	66.7	83.3
3_____	16	6.3	0	93.8	100.0
Total or average_____	52	26.0	19.4	81.2	94.4

DISCUSSION

The significant results in tables 2 and 3 and summarized in table 4 are the percentages of agreement between the various tests and the bacteriological findings.

In tables 2 and 3, particular interest centers on the results with commercial pullorum no. 1. The birds used were chiefly pullets. Eight of the nine cases examined post mortem showed no macroscopic lesions of pullorum disease, and *Salmonella pullorum* was isolated from only 4 of the 9 birds. With young fowls it is more difficult to harmonize the serological and bacteriological results than with more mature ones. The corroborative evidence of the tube test in this group gives support to the inference that pullorum infection, though undiscovered in some birds, probably lurked in their bodies.

As already noted, macroscopic lesions of pullorum disease in reactors are sometimes lacking, particularly in young fowls, even when the infection may be demonstrable by bacteriological procedure. Therefore a diagnosis eliminating pullorum disease on the basis of no lesions cannot be made with assurance.

In table 4 there is seen to be excellent average agreement, 94.4 percent, between the rapid whole-blood agglutination test and the tube test, thus supporting the reliability of the rapid test. There is also good agreement, 81.2 percent, between the rapid whole-blood agglutination test and the bacteriological findings. The results obtained by the pullorin test show a much lower percentage of agreement with the bacteriological findings and the tube agglutination test. The evidence thus obtained indicates that the rapid whole-blood test is a more reliable diagnostic agent for pullorum disease than the pullorin test.

SUMMARY AND CONCLUSIONS

Each of four commercial poultry flocks was tested for pullorum disease by the stained-antigen, rapid whole-blood agglutination test and the pullorin test. In addition, the tube agglutination test was used post mortem on the serum of birds from three of the flocks, as a check on the reliability of the rapid whole-blood method. Specimen birds were obtained from these flocks, examined post mortem, studied

bacteriologically, and the findings compared with the test findings. In every group the agreement between the rapid whole-blood agglutination test and the bacteriological findings was more favorable than that between the pullorin test and the bacteriological findings. The results of the tube agglutination method, which is of recognized dependability, supported the reliability of the rapid whole-blood agglutination test.

Although the experiments here recorded are of limited scope, the evidence obtained is in accord with the views expressed by other workers, that the pullorin test is not so satisfactory a means of diagnosing pullorum disease as the rapid whole-blood agglutination test.

THE DISTRIBUTION AND CONDITION OF NITROGEN IN THREE HORIZONS OF A DIFFERENTIALLY FERTILIZED HAGERSTOWN CLAY LOAM SOIL PLANTED TO APPLE TREES IN METAL CYLINDERS [1]

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INTRODUCTION

In a recent paper (*15*) [2] the writer reported the utilization and recovery of nitrogen, phosphorus, and potassium by apple trees grown in metal cylinders for a period of 6½ years. These trees received (each spring) during the last 3 years of growth different combinations of the pure salts sodium nitrate, monocalcium phosphate, and potassium sulphate. It was shown that the ratio in which nitrogen (as N), phosphorus (as P_2O_5), and potassium (as K_2O) were absorbed from the added salts ($NaNO_3$, $CaH_4(PO_4)_2$, and K_2SO_4) by those trees optimum with respect to growth and reproduction, that is, the trees receiving the NPK and NP treatments, was 3:0.3:1.5 as compared with a 3:8:4 ratio actually applied. This latter ratio was based on the current orchard practice. The great divergence between these ratios indicated the need for information as to the condition of the added nitrogen, phosphorus, and potassium in the soil in order to determine the extent to which these "theoretical" quantities and ratios should be modified as a result of the changes produced by the interaction of the added salts with the soil. The present investigation reports the status of the nitrogen in the three soil horizons in the cylinders from which the trees referred to above were removed.

METHODS OF EXPERIMENTATION

Inasmuch as the detailed plan of this experiment has been reported elsewhere (*3, 14, 16*) a brief outline only is necessary.

The soil, the analysis of which has been recorded (*11*), was formed in place by the weathering of limestone to the lower Silurian formations, and is of Trenton origin. The excavation was made on a strip of land 110 by 11 feet adjacent to the college experimental orchard. The history of this land indicates that except for the droppings of cattle no dressings of fertilizer had ever been applied. It may, therefore, be described as a virgin forest soil. The mechanical analysis (*11*) suggests that the surface soil consists of a heavy silt loam underlain by a clay loam which becomes heavier in texture as the depth increases.

The soil was excavated from this strip by a scoop shovel. The layers from each of three horizons, viz, surface (0 to 7 inches), subsurface (7 to 21 inches), and subsoil (21 to 53 inches), were kept separate and each was thoroughly mixed and weighed. The total

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(845)

weight of the horizons was 54,180, 109,200, and 249,000 pounds, respectively. Inasmuch as there were 42 cylinders, an equal distribution (by weight) of each layer among the cylinders would require the following quantities of soil to be added to each of the cylinders: Subsoil, 5,930 pounds; subsurface soil, 2,600 pounds; and surface soil, 1,290 pounds. This equal distribution was effected by ascertaining the weight of each of the respective horizons required to fill a steel wheelbarrow similar to those used in highway construction work. Such wheelbarrows were used in filling the cylinders. Following the addition of each wheelbarrow load, uniformity in density of the soil was secured by means of heavy wooden mallets fitted with 3-inch cast-iron pipe handles 5 feet in length. The process of filling the cylinders was completed in the spring of 1920. Uniformity with respect to the nitrogen, phosphorus, and potassium content of the soil in the cylinders at this stage was established by analysis. The mean of 30 determinations for total nitrogen in the original soil is given in the first line of table 1. Any departure from these values greater than the error of analysis (± 0.0001 percent) must be attributed to causes resulting from differences in treatment.

The trees were planted in the spring of 1922. Up to the spring of 1924 the system of culture was similar in all cylinders. This consisted of green manuring with buckwheat and rye principally. In May 1924 half the cylinders were seeded with a mixture of Kentucky bluegrass, *Poa pratensis* L., and timothy, *Phleum pratense* L. These cylinders are designated "cylinders under sod." In the remaining half of the cylinders a tillage system was adopted. These latter cylinders are designated "cylinders under cultivation." A distinction must be noted with respect to the additions of nitrogen from 1925 until the end of the experiment in 1927. During these last 3 years of the experiment the cylinders under cultivation received 15.9 grams more nitrogen than the cylinders under sod. The reason for this is that it was then considered necessary to add equal amounts of organic matter to all the cylinders under cultivation. This was accomplished by growing rye outside the cylinders. For further details the paper by Anthony and Clarke (*3, p. 251*) should be consulted. All trees were allowed to grow without the addition of any mineral fertilizer until the spring of 1925, at which time differential treatment with different combinations of sodium nitrate, mono-calcium phosphate, and potassium sulphate was commenced. It is important to note that the conditions of this experiment preclude any erosion by water and practically none by wind.

The sodium nitrate was added according to the following schedule:

	Grams
April 18, 1925	906
May 3, 1926	45
June 7, 1926	453
June 20, 1926	408
May 5, 1927	337
May 18, 1927	338
June 10, 1927	337
Total	2,824

This total of 2,824 g of sodium nitrate is equivalent to 465.5 grams of elemental nitrogen.

During the period from September 20 to 28, 1927, the trees were dup up and soil samples representative of the three horizons were taken, by the method of successive quartering, from each of the cylinders from which the trees had been removed. These samples were dried at 75° C. and then sieved through a 1-millimeter sieve (*4*) and stored in glass jars in the dark. Analyses of the trees have already been reported (*14*).

Total nitrogen was determined by the Kjeldahl-Gunning method to include the nitrogen of nitrates, a 15-g charge being used for the surface soil and 30-g for the subsurface and subsoil (*4*). Nitric nitrogen was determined by the Devarda alloy method, a 200-g charge being used (*1*). The analytical data in the tables are the means of closely agreeing triplicate determinations. The quantities of ammonia nitrogen and nitrous nitrogen in all horizons were insignificant. All calculations are made on a moisture-free basis.

EXPERIMENTAL DATA

Table 1 gives the percentage and absolute amounts of total nitrogen in each of the three soil horizons; that is, the 0 to 7 inch, the 7 to 21 inch, and the 21 to 53 inch.

TABLE 1.—*Percentages and absolute amounts of total nitrogen in the respective horizons before the trees were planted and at the end of the experiment*

Treatment	Total nitrogen			Absolute amount total nitrogen			
	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Total (0–53 inches)
	Percent	*Percent*	*Percent*	*Grams*	*Grams*	*Grams*	*Grams*
Soil before trees were planted	0.08650	0.05003	0.03523	506.1	589.7	946.8	2,042.6
Sod:							
Check	.08219	.04600	.03400	477.9	542.5	914.5	1,934.9
PK	.08200	.04700	.03498	479.8	554.3	941.4	1,975.5
NPK	.08200	.05160	.03900	478.6	613.2	1,049.0	2,140.8
NP	.08180	.05250	.03900	478.6	619.1	1,049.0	2,146.7
NK	.08200	.05500	.04040	479.8	648.6	1,083.0	2,211.4
N	.08200	.05200	.04000	479.8	625.0	1,075.9	2,180.7
Cultivation:							
Check	.07110	.05100	.03445	415.9	601.5	926.6	1,944.0
PK	.07485	.05000	.03571	436.4	590.4	960.5	1,987.3
NPK	.07800	.05290	.04310	456.4	620.8	1,146.6	2,223.8
NP	.07900	.05230	.04250	462.2	614.4	1,142.9	2,219.5
NK	.07820	.05500	.04350	457.6	648.8	1,170.1	2,276.5
N	.07890	.05200	.04200	461.6	620.2	1,131.9	2,213.7

Table 2 gives the percentage and absolute amounts of nitric nitrogen and of nonnitric nitrogen. The latter values were obtained by difference between the total nitrogen and the nitric nitrogen.

TABLE 2.—*Percentage and absolute amounts of nitric and nonnitric nitrogen in the respective horizons before trees were planted and at the end of the experiment*

Treatment	Nitric nitrogen			Absolute amount nitric nitrogen			
	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Total (0–53 inches)
	Percent	*Percent*	*Percent*	*Grams*	*Grams*	*Grams*	*Grams*
Soil before trees were planted___	0.00170	0.00053	0.00023	9.9	6.3	6.2	22.4
Sod:							
Check_____	.00069	.00080	.00060	4.0	9.4	16.1	29.5
PK_____	.00070	.00100	.00066	4.1	11.8	17.7	33.6
NPK_____	.00220	.00760	.00450	12.9	89.6	121.0	223.5
NP_____	.00200	.00720	.00500	11.7	84.9	133.5	230.1
NK_____	.00290	.00900	.00560	16.9	106.1	147.0	270.0
N_____	.00240	.00820	.00550	14.0	96.7	147.9	258.6
Cultivation:							
Check_____	.00110	.00100	.00065	6.4	11.8	17.5	35.7
PK_____	.00085	.00120	.00071	3.5	14.1	19.1	36.7
NPK_____	.00210	.00640	.00410	12.3	72.4	111.0	195.7
NP_____	.00200	.00610	.00460	11.7	69.6	123.5	204.8
NK_____	.00270	.00690	.00590	15.8	81.5	158.7	256.0
N_____	.00290	.00760	.00500	16.9	89.5	136.7	243.1

Treatment	Nonnitric nitrogen			Absolute amount nonnitric nitrogen			
	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Surface (0–7 inches)	Subsurface (7–21 inches)	Subsoil (21–53 inches)	Total (0–53 inches)
	Percent	*Percent*	*Percent*	*Grams*	*Grams*	*Grams*	*Grams*
Soil before trees were planted___	0.0848	0.0495	0.0350	496.2	583.4	940.6	2,020.2
Sod:							
Check_____	.0815	.0452	.0334	473.9	533.1	898.4	1,905.4
PK_____	.0813	.0460	.0343	475.7	542.5	923.7	1,941.9
NPK_____	.0798	.0444	.0345	468.9	523.6	928.0	1,918.5
NP_____	.0798	.0453	.0340	466.9	534.2	915.5	1,916.6
NK_____	.0791	.0460	.0348	462.9	542.5	936.0	1,941.4
N_____	.0796	.0448	.0345	465.8	528.3	928.0	1,922.1
Cultivation:							
Check_____	.0700	.0500	.0338	409.5	589.7	909.1	1,908.3
PK_____	.0740	.0488	.0350	432.9	576.2	941.4	1,950.5
NPK_____	.0759	.0465	.0390	444.1	548.4	1,035.6	2,028.1
NP_____	.0770	.0462	.0379	450.5	544.8	1,019.4	2,014.7
NK_____	.0755	.0481	.0376	441.8	567.3	1,011.4	2,020.5
N_____	.0760	.0450	.0370	444.7	530.7	995.2	1,970.6

DISCUSSION OF DATA

TOTAL NITROGEN

The data in table 1 indicate that at the end of the experiment the total nitrogen content of the surface soil in all cylinders under sod was slightly greater than that in cylinders under cultivation, although, as has already been pointed out, the cylinders under cultivation had received 15.9 grams more nitrogen than the corresponding cylinders under sod. The differences between the total nitrogen content of corresponding cylinders under the two systems in the respective horizons are shown in table 3.

The data in table 3 do not take into account the nitrogen removed by the trees and, in sod, by the grass also. The disappearance of nitrogen (as total nitrogen) when the amounts removed by the trees are taken into account is shown in table 4. This disappearance is called by some investigators "the apparent loss of nitrogen."

TABLE 3.—*Difference* [a] *(in grams) between the amounts of total nitrogen present in the 3 horizons of the 2 systems*

Horizon	Check	PK	NPK	NP	NK	N
Surface (0-7 inches)	+62.0	+43.4	+23.4	+16.4	+22.2	+18.2
Subsurface (7-21 inches)	−59.0	−36.1	−7.6	+4.7	0	+4.8
Subsoil (21-53 inches)	−12.1	−19.1	−97.6	−93.9	−87.1	−56.0
Total (0-53 inches)	−9.1	−11.8	−81.8	−72.8	−64.9	−33.0

[a] The sign indicates the amount in grams by which the total nitrogen under sod is greater than (+) or less than (−) under cultivation.

TABLE 4.—*Nitrogen disappearance (grams) calculated on the total nitrogen of the soil in the whole layer (0 to 53 inches) at the end of the experiment*

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) Amount N present in soil before experiment	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6
(2) Amount N added in NaNO₃ (=465.0 g)+seeds (=2.5 g)	2.5	2.5	467.5	467.5	467.5	467.5
(3) Amount N from (1)+(2)	2,045.1	2,045.1	2,510.1	2,510.1	2,510.1	2,510.1
(4) Amount N found	1,934.9	1,975.5	2,142.0	2,146.7	2,211.4	2,180.7
(5) Loss of N from soil	−110.2	−69.6	−368.1	−363.4	−298.7	−329.4
(6) Total amount N absorbed by trees during growth (tops and roots)	36.5	56.8	201.4	190.4	133.4	124.3
(7) Disappearance of N by leaching and possibly as gaseous N during the 6½ years of the experiment	−73.7	−12.8	−166.7	−173.0	−165.3	−205.1

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) Amount N present in soil before experiment	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6	2,042.6
(2) Amount N added in NaNO₃ (=465.0 g)+seeds (=2.5 g)	19.4	19.4	483.4	483.4	483.4	483.4
(3) Amount N from (1)+(2)	2,062.0	2,062.0	2,526.0	2,526.0	2,526.0	2,526.0
(4) Amount N found	1,944.0	1,987.3	2,223.8	2,219.5	2,276.5	2,213.7
(5) Loss of N from soil	−118.0	−74.7	−302.2	−306.5	−249.5	−312.3
(6) Total amount N absorbed by trees during growth (tops and roots)	53.5	63.4	180.9	170.3	131.8	121.3
(7) Disappearance of N by leaching and possibly as gaseous N during the 6½ years of the experiment	−64.5	−11.3	−121.3	−136.2	−117.7	−191.0

Considering the whole depth 0 to 53 inches, the losses from the nitrogen-treated cylinders are greater under sod than under cultivation. The amounts by which the losses (in grams) under the former system exceed those under the latter are: NPK, 45.4; NP, 36.8; NK, 47.6; and N, 14.1 These differences appear to be related to the accretion of nonnitric nitrogen in the subsoil of the cylinders under cultivation. The net result is a gain in nonnitric nitrogen when calculated on the whole depth (0 to 53 inches) in the treated cylinders under cultivation as compared with those under sod. This point is discussed later.

Line 7 of table 4 gives the losses by leaching and possibly as gaseous nitrogen (i.e., the so-called "nitrogen balance") for the whole soil layer (0 to 53 inches). The same data calculated for the surface 0 to 7 inches and subsurface 0 to 21 inches only are shown in table 5.

TABLE 5.—*Nitrogen balance (in grams) calculated to less than full depth*

Horizon	Check	PK	NPK	NP	NK	N
0 to 7 inches:						
Under sod	+5.8	+34.0	−292.4	−304.6	−360.4	−369.5
Under cultivation	−56.1	−25.7	−352.2	−357.0	−400.1	−406.6
0 to 21 inches:						
Under sod	−41.4	−7.4	−268.9	−275.2	−301.5	−334.2
Under cultivation	−44.3	−25.0	−321.1	−332.3	−341.0	−376.1

The nitrogen balance is seen to vary with the depth of soil upon which the calculations are based. In the 0- to 7-inch layer, a nitrogen gain is indicated in the cylinders under sod to which no mineral nitrogen was added. But if the calculations are based on the 0- to 21-inch layer or on the whole depth, 0- to 53-inch layer, losses of nitrogen are definitely established. The larger losses shown in the nitrated cylinders in the 0- to 7-inch and 0- to 21-inch layers as compared with the 0- to 53-inch layer appear to be only an expression of the fact that the quantity of nitrates (nitric nitrogen) becomes greater with depth.

More information with respect to the status of the nitrogen is obtained by considering the nitric and nonnitric fractions of the total nitrogen separately. These are shown in table 2.

NITRIC NITROGEN (NITRATES)

MOBILITY OF ADDED NITRIC NITROGEN

Table 2 shows that the nitric nitrogen calculated on a percentage basis, i.e., the concentration of nitrates, is greater in the subsurface than in either of the other horizons. The absolute amount of nitric nitrogen, however, is greater in the subsoil in all cases. The last application of $NaNO_3$ (167 grams nitrogen) was made in the spring of 1927, $4\frac{1}{2}$ months before the trees were removed. Presumably, therefore, this increased concentration of nitric nitrogen in the subsurface is merely an expression of the movement of nitrogen as nitric nitrogen from this last application, and when taken in conjunction with the data for the nitric nitrogen in the check and PK cylinders, suggests that the greater part of the last application was still in the 7- to 21-inch layer at the conclusion of the experiment.

NITRIC NITROGEN UNDER SOD AND CULTIVATION

It will be recalled that each of the cylinders under cultivation received 15.9 grams more nitrogen in the form of rye cover crop than the cylinders under sod. Now the concentration of nitric nitrogen in the whole depth (0 to 53 inches) of the check cylinder under cultivation is 35.7 grams and that of the cylinder under sod is 29.5 grams as compared with 22.4 grams in the original soil. However, in the cylinders which received mineral nitrogen in addition to that introduced by the green manures (the NPK, NP, NK, and N cylinders) the concentration of nitric nitrogen in the whole depth is much greater in all cases in the cylinders under sod. This may be only another expression of the difference in the status of the soil nitrogen in the three horizons under sod and cultivation previously referred to in the discussion of the disappearance of nitrogen (as total N) by leaching and possibly as gaseous nitrogen. This will be brought out more clearly in the discussion of the nonnitric nitrogen fraction.

<center>An Inventory of Nitric Nitrogen</center>

A more complete picture of the status of the nitrates may be obtained from the inventory of nitric nitrogen shown in table 6, in which account has been taken of the nitric nitrogen equivalent to that absorbed by the trees under the different treatments from the added NaNO$_3$.

TABLE 6.—*Inventory of nitric nitrogen (in grains) at end of experiment (0 to 53 inches)*

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) N added to each cylinder in the form of NaNO$_3$	0	0	465.5	465.5	465.5	465.5
(2) N absorbed by each tree during growth and also (in sod) by the grass	36.5	56.8	201.4	190.4	133.4	124.3
(3) N absorbed by each tree from added NaNO$_3$	0	0	132.7	132.4	91.9	83.6
(4) Theoretical amount of N expected in soil			332.8	333.1	373.6	381.9
(5) N found	29.5	33.6	223.5	230.1	270.0	258.6
(6) Disappearance of nitric N during the 6½ years of the experiment			109.3	103.0	103.6	123.3

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) N added to each cylinder in the form of NaNO$_3$	0	0	465.5	465.5	465.5	465.5
(2) N absorbed by each tree during growth and also (in sod) by the grass	53.5	63.4	180.9	170.3	131.8	121.3
(3) N absorbed by each tree from added NaNO$_3$	0	0	117.5	93.2	78.3	67.8
(4) Theoretical amount of N expected in soil			348.0	372.3	387.2	397.7
(5) N found	35.7	37.0	195.7	204.8	256.0	243.1
(6) Disappearance of nitric N during the 6½ years of the experiment			152.3	167.5	131.2	154.6

The nitric nitrogen absorbed by the trees (table 6, line 3) was obtained in a manner similar to that described in an earlier paper (*15, pp. 570–573*). The values in line 3 represent the difference between the amount of nitrogen absorbed by a tree which received additions of another element (or other elements) than nitrogen and a tree from which nitrogen was omitted. The values so obtained may not be mathematically exact, inasmuch as the Wirkungswert (effect factor) of an element may not be the same in the presence of another factor or factors as when that factor operates alone. Nevertheless, there are numerous experiments that lend support to Mitscherlich's contention (*7*) that the Wirkungswert of an element may be fairly constant, especially under the controlled conditions of such an experiment as the present one. The method is believed to be sufficiently accurate to bring out more clearly any characteristic differences in the status of the nitric nitrogen of the respective treatments and especially with respect to differences between the two cultural systems. The procedure adopted may be more readily understood from the following algebraical analysis:

Let a = amount of nitrogen present in the soil of each cylinder before the trees were planted.

Let x=amount of nitrogen added to each cylinder in rain and snowfall.
Let y=amount of nitrogen added to each cylinder under cultivation in the form of organic matter (buckwheat and rye).
Let z=the total amount of sodium nitrate added to each of the "nitrated" cylinders.

Now the nitrogen-treated trees have obtained the nitrogen absorbed by them from all of the foregoing sources, and the trees which did not receive mineral nitrogen ($NaNO_3$) additions absorbed nitrogen from all of these sources except z.

For greater simplicity and clarity, let us first of all consider the absorption of nitrogen from only two of the trees on which chemical analyses were made; namely, the NPK and the PK trees, both under the tillage system.

The amount of nitrogen absorbed by the NPK tree during the whole period of its growth will be some fraction of $a+x+y+z$. Let this fraction be designated k $(a+x+y+z)$. Similarly, the amount of nitrogen absorbed by the PK tree during the whole period of its growth will be some fraction of $(a+x+y)$, which will be designated $k'(a+x+y)$.

$$\text{Let } \frac{1}{s}=k(a+x+y+z) \tag{1}$$

$$\text{Let } \frac{1}{r}=k'(a+x+y) \tag{2}$$

Then, by subtraction

$$\frac{1}{s}-\frac{1}{r}=k(a+x+y+z)-k'(a+x+y) \tag{3}$$

Now, if $k(a+x+y)$ is equal to or very nearly equal to $k'(a+x+y)$,

$$\text{then, from (3), } \frac{1}{s}-\frac{1}{r}=kz \tag{4}$$

or, expressed in words, the fraction of the nitrogen added to the NPK tree in the form of sodium nitrate is obtained by difference between the total amount of nitrogen absorbed by that tree and that absorbed by the PK tree.

In the present experiment the trees were grown without mineral salt additions for the first 4 years. The factor z then of equation (1) does not enter into the picture during this period.

For the purpose of the present analysis the difference between the quantities $k(a+x+y)$ must be very small as compared with the quantity kz. In further support of this contention may be cited the mathematical analysis given by the writer in an earlier paper (*13*).

The amounts of nitrogen applied as sodium nitrate were 149 g in 1925, 149 g in 1926, and 167 g in 1927, a total of 465 g. A comparison of these quantities of added nitrogen with the quantities actually present (table 6, line 5) shows that all of the "nitrated" cylinders contained at the end of the experiment more than enough nitrates to account for the amount (167 g) added the last year of the experiment, 4½ months before the samples were taken in the fall of 1927, and in addition a considerable portion of the nitrogen added in the second application in the spring of 1926. The total precipitation during the period between one application and the next was: May 18, 1925, to May 2, 1926, 31.8 inches; May 3, 1926,

to May 4, 1927, 44 inches; May 5, 1927, to September 20, 1927, 18.6 inches. In addition, 2 inches of artificial watering was applied in May 1926 and 1 inch in August 1927.

The Rothamsted experiments (*9*) on the losses of nitrogen in the drainage waters from a plot of arable land kept free of vegetation since 1870, which received no artificial additions of nitrogen, are frequently cited in support of the view that nitrates are readily leached from soils. At the end of 47 years the amounts of nitric nitrogen found in the drainage waters were equal to the total losses of nitrogen from the soil. The rate of loss was equal to 40 pounds per annum per acre in the earlier years and below 25 pounds per annum per acre in the later years.

In some soil types in Tennessee (*8*), however, when nitrogen was applied as sodium nitrate to lysimeters kept bare of vegetation, the leaching (outgo) of nitrogen in 2½ years was as low as 34 percent. Mooers and MacIntire attribute this relatively small loss to the influence of the heavy clay subsoil into which the nitrate ion passes as magnesium and calcium nitrates through base exchange.[3]

The investigations of De Sornay (*10*) also indicate that nitrates may remain in the soil available to plants for long periods, moving upward or downward according to moisture conditions. The upward capillary attraction was found to be much more rapid than the downward displacement by rain. De Sornay reports that Demolon and Brouet at Aisne found, in an uncropped, light, sandy garden soil, that after a rainfall of 9.8 inches during a period of 2 months more than one half of the added sodium nitrate remained in the first 8 inches.

More recently Ames (*2*) has reported that during the period 1928-30 the nitric nitrogen content of the soil under corn or soybeans never exceeded 50 pounds per acre in the surface 6⅔ inches, but in 1931, after a year of drought, the nitric nitrogen content reached 300 pounds per acre.

The problem is summed up by MacIntire [4] as follows:

It is exceedingly difficult to make an unqualified statement as to the fate of added nitrogen. This will vary with the soil, alkalinity or acidity, climatic conditions, the amount of added nitrogen, absence or presence of growing plants and the type of these, and periodicity of rainfall, together with the very important fact of depth and type of subsoil.

In the present experiment the significant fact is that the disappearance of nitric nitrogen is greater in all the cylinders under cultivation to which mineral nitrogen was added than under the corresponding cylinders under sod.

NONNITRIC NITROGEN

The nonnitric nitrogen consists of (1) nitrogenous organic material potentially "available" but not yet decomposed; (2) the humus nitrogen characterized by marked stability; (3) the nitrogen synthesized by the micro-organisms; (4) ammonia nitrogen absorbed by the colloidal soil complex.

The apparent gain or loss in integral numbers with respect to the nonnitric nitrogen is given in table 7. The quantities of nitrogen shown in line 4, that is, the amount of nitrogen absorbed by the trees from sources other than the nitrogen added as $NaNO_3$, were obtained

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[3] MacIntire, W. H. Private communication.
[4] MacIntire, W. H. See footnote 3.

by using the quantities of nitrogen absorbed by corresponding treatments to which no nitrogen was added. For example, the amount of nitrogen absorbed by the NPK tree in sod from sources other than that equivalent to the added $NaNO_3$ was obtained by difference between this quantity and that absorbed by the PK tree also growing in sod. For reasons already given these values have no pretention to mathematical exactness. But in the present analysis they serve for all practical purposes to furnish a picture of the changes in nonnitric nitrogen of the original soil as a result of the various treatments under the two culture systems.

TABLE 7.—*Gain or loss (grams) of nonnitric nitrogen from whole depth of soil (0 to 53 inches) by the end of the experiment*

Item	Sod					
	Check	PK	NPK	NP	NK	N
(1) Nonnitric N before experiment	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2
(2) Nonnitric N at end of experiment	1,905.4	1,941.9	1,918.5	1,916.6	1,941.4	1,922.1
(3) Actual loss or gain by soil	−114.8	−78.3	−101.7	−103.6	−78.8	−98.1
(4) N absorbed per tree from sources other than the added $NaNO_3$	36.5	57.0	57.0	49.9	37.4	37.4
(5) Apparent total loss or gain during the 6½ years of the experiment	−78.3	−21.3	−44.7	−53.7	−41.4	−60.7

Item	Cultivation					
	Check	PK	NPK	NP	NK	N
(1) Nonnitric N before experiment	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2	2,020.2
(2) Nonnitric N at end of experiment	1,908.4	1,950.5	2,028.1	2,014.7	2,020.5	1,970.6
(3) Actual loss or gain by soil	−111.9	−69.7	+7.9	−5.5	+.3	−49.6
(4) N absorbed per tree from sources other than the added $NaNO_3$	53.5	63.4	63.4	77.1	53.5	53.5
(5) Apparent total loss or gain during the 6½ years of the experiment.	−58.4	−6.3	+71.3	+71.6	+53.8	+3.9

In both culture systems there is a disappearance of nonnitric nitrogen in the cylinders which received no mineral nitrogen additions (check and PK cylinders), the loss being greater under sod. But, whereas relatively large accretions of nonnitric nitrogen have occurred in the NPK, NP, NK, and N cylinders under cultivation, losses of nonnitric nitrogen have occurred from the corresponding cylinders under sod. These differences are much greater than can be accounted for by sampling or analytical errors. Although uniformity with respect to content of nitrogen, phosphorus, and potassium was established in the cylinders before the experiment began, there may have existed differences in respect to the physical condition that would preclude a definite and unqualified interpretation of the differences existing with respect to the condition of the nitrogen under sod and cultivation. Three explanations may be advanced:

(1) Assimilation of nitrogen added as $NaNO_3$ by micro-organisms. But the difficulty lies in explaining why assimilation should have occurred in the cylinders under cultivation and not in those under sod. Carbon dioxide accumulation under grass (*12*) may be the differential factor. Some nitrogen would appear to have been brought up and immobilized in the surface soil by the grass roots in the sod system, inasmuch as the quantity of nitrogen (as total and nonnitric nitrogen)

of the surface soil is higher in all cases under grass than under cultivation. But the results for the whole depth (0 to 53 inches) show that this explanation is insufficient to account for the entire difference in the amount of nitrogen in corresponding cylinders in the two systems.

(2) The peptization of nitrogenous organic material by $NaNO_3$ in the first horizon and subsequent leaching. Hardpan formation was particularly noticeable in the nitrogen-treated cylinders under cultivation. Cracks and fissures, therefore, may have assisted the downward movement.

(3) The greater root system under cultivation. Except in one tree (NPK) the root systems were larger in the trees grown under cultivation. The weights of the root systems are given in table 8.

TABLE 8.—*Weights in grams of the respective root systems in soils in sod and under cultivation*

Condition	Check	PK	NPK	NP	NK	N
Sod	9, 950	8, 325	13, 040	12, 730	11, 090	10, 366
Cultivation	10, 870	10, 695	12, 395	13, 075	11, 700	12, 275

The higher nitrogen content of the soil under cultivation may have arisen from sloughed-off portions of fibrous roots that might have been incorporated in the soil in the process of preparation for analysis.

The extensive literature of the problem of the mineralization of nitrogen in the soil has been reviewed by Lemoigne (*5*), Lyon (*6*), and Waksman (*17*). The results presented in this paper suggest the desirability of further investigation of the problem.

SUMMARY

The distribution of total nitrogen and also of the nitric and nonnitric fractions in three horizons, viz, 0 to 7 inches, 7 to 21 inches, and 21 to 53 inches, of a Hagerstown clay loam soil contained in cylinders planted to apple trees and treated with different combinations of sodium nitrate, monocalcium phosphate, and potassium sulphate are given in percentage and in absolute amounts.

In all treatments the total nitrogen of the surface soil under sod was somewhat greater than under cultivation. In the subsurface the differences in total nitrogen were small except in the check cylinders under sod, in which it was less than under tillage. In all treatments the total nitrogen of the subsoil was greater in the cylinders under cultivation than in the corresponding cylinders under sod. For the whole depth (0 to 53 inches), the total nitrogen at the end of the experiment was greater in all cylinders under cultivation than in those under sod.

The disappearance of nitrogen (as total nitrogen) by leaching, and possibly as gaseous nitrogen, was greater under sod than under cultivation in all cases.

The movement of nitric nitrogen is discussed. It is concluded that leaching of nitrates from this heavy soil was not very rapid.

The disappearance of nitric nitrogen, when account has been taken of the nitric nitrogen absorbed by the trees equivalent to that added as $NaNO_3$, was greater in all nitrated cylinders under cultivation than

in corresponding cylinders under sod. This difference is accounted for by an accretion of nitrogen as nonnitric nitrogen in the subsoil under cultivation but not in that under sod.

Results with respect to nitrogen gains or losses based on the soil to a depth of 53 inches are very different from those based on the 0- to 7-inch or 0- to 21-inch depths.

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LIFE HISTORY OF THE HAIRY-ROOT ORGANISM IN RELATION TO ITS PATHOGENESIS ON NURSERY APPLE TREES [1]

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INTRODUCTION

In studies of infectious hairy root carried on during the last 4 years, special consideration has been given to the life history of the causal organism in relation to its pathogenesis on nursery apple trees. This disease has been so prevalent on grafted apple trees in the nursery that it has become of considerable economic importance. The causal organism, *Phytomonas rhizogenes* Riker et al., has recently been differentiated by Riker and his associates *(26)*[3] as a new species distinct from that causing crown gall, *P. tumefaciens* (Smith and Town.) Bergey et al. Previously it had been considered an apple strain of the crown-gall organism by Smith et al. *(36)* and more completely developed as such by Siegler *(33, 34)*.

The name "hairy root" appears to have been first introduced into literature by Stewart, Rolfs, and Hall *(37)*. Following the earlier work of Hedgcock *(11)* on the identity of the complex of malformations occurring on apple, a number of well-known papers appeared. Recently a number of diseases have been separated from this complex on the basis of cause, viz, (1) infectious hairy root, (2) crown gall, (3) wound overgrowth, and (4) nonparasitic hairy root. Of these, infectious hairy root is now perhaps the most important from the economic standpoint.

The host range of the hairy-root organism is little understood. Up to the present time the writer has found it reported under natural conditions only on apple. However, cross-inoculation studies by various workers, including Smith et al. *(36)*, Riker et al. *(25)*, Brown *(6)*, Banfield *(3)*, and Riker et al. *(26)*, have demonstrated the pathogenicity of these bacteria on such plants as sugar beet *(Beta vulgaris* L.) quince *(Cydonia oblonga* Mill.), rose *(Rosa setigera* Michx.), honeysuckle *(Lonicera morrowi* Gray), Paris daisy *(Chrysanthemum frutescens* L.), balsam *(Impatiens balsamina* L.), bryophyllum *(Bryophyllum pinnatum* Kurz), red raspberry *(Rubus idaeus* L.), bean *(Phaseolus vulgaris* L.), and sedum *(Sedum spectabile* Bor.).

Hairy root appears to be widely distributed. Hedgcock *(11)*, who reported crown gall from all the States of the United States except

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Nevada, stated that "the forms of the disease known as hairy root have been found as widely disseminated as crown gall on apple trees in nurseries and orchards in the United States." He also reported forms of hairy root from Germany, Netherlands, France, Canada, and New Zealand. Doidge (8) recorded the presence of the disease in South Africa, and Noble (18) in Australia. The disease seems widely disseminated, but the difficulties of diagnosis raise a question as to the accuracy of some of the reports.

The economic importance of infectious hairy root, although considerable, is hard to estimate since no information is available as to how much of the loss due to malformations may be attributed to this disease. Studies in which the writer has participated for more than 4 years, in nurseries from Wisconsin to Oklahoma, reveal that while other difficulties in this complex have been largely eliminated hairy root still remains a factor of considerable importance in certain places.

Control measures at the present time are only partially satisfactory. Von Schrenk and Hedgcock (39) laid the foundation for the most successful of later attempts to control the various malformations at the unions of piece-root grafts when they noted that these enlargements usually appeared at the graft union, that using root and scion pieces of nearly the same diameter reduced overgrowths, and that wrapping the unions with certain materials such as cloth and rubber considerably increased the percentage of smooth trees over those wrapped with other materials. Since the publication of their report many control measures have been suggested by different workers, as, for example, Melhus and Maney (15), Wormald and Grubb (41), Riker and Keitt (30), Waite and Siegler (40), Melhus, Muncie, and Fisk (16), and Maney and Pickett (14). Additional studies by Riker and his associates (21, 24, 27, 31) have repeatedly demonstrated the value of nurseryman's tape, a special kind of adhesive plaster. Since the discovery that this tape prevents a large percentage of union malformations without producing any ill effects, it has come into common use. Among the nurserymen there is a feeling that the saving in handling the grafts bound with this wrapper more than pays for its extra cost. In addition, there is a considerable increase in the number of clean trees. The use of nurseryman's tape has eliminated on an average more than half of the various outgrowths at the union. Those that remain are mostly hairy root.

The persistence of malformations, chiefly of the hairy-root type, caused by *Phytomonas rhizogenes*, appeared to warrant a study of the life history of the causal organism in relation to pathogenesis. It was hoped that such a study would not only increase the available information on the fundamental activity of this organism but would also define critical points at which the application of control measures might be more effective.

IDENTITY OF HAIRY ROOT

Before studying the pathogenic life history of infectious hairy root it seemed desirable to make a reexamination of the identity of hairy root as contrasted with crown gall, wound overgrowth, and other malformations occurring on the underground parts of nursery apple trees.

The isolation of the hairy-root organism was attempted from a variety of malformations on Wealthy apple trees collected at random

at digging time from the experimental plots at Madison, Wis., and Topeka, Kans. It was found from the outward appearance and interior structure that these overgrowths could be roughly classified into three groups: (1) Convoluted, with roots, characterized by a hard vascular interior and a soft exterior layer of variable thickness that turned brown rapidly when exposed to the air; (2) convoluted, without roots, identical with the foregoing except for the absence of roots; and (3) undulated, without roots and, unlike the other enlargements, chiefly made up of whitish vascular cortex. The enlargements differed considerably in appearance and in the number of emerging roots, making them difficult to classify. Nevertheless it seemed important to attempt isolations from such specimens.

The method of isolation was as follows: From a specimen of each type of overgrowth several cubes of overgrowth tissue, approximately 3 mm on a side, were removed from the soft tissue, if present, under aseptic conditions and dropped into three Petri dishes each containing 1 cc of sterile distilled water. A sterilized scalpel was used to macerate the tissue in the water. After an interval of about 10 minutes dilutions were made from each plate to three successive Petri dishes. Patel's (*19*) bile agar was then added. The poured plates were incubated at 28° C. and examined after 1 week. The identity of the bacteria that appeared was determined after inoculations on sedum and apple and after cultural examination, as suggested by A. J. Riker, on sodium selenite agar. A summary of these studies is given in table 1, showing that a majority of the convoluted enlargements with roots were infectious hairy root and that a considerable part of the convoluted enlargements without roots were also hairy root. The undulated enlargements did not yield the infectious agent and in all probability they were nonparasitic overgrowths. Since the experiment described below showed the isolation technic to be reasonably accurate, it appears that many of the specimens contained tissue not primarily stimulated by the hairy-root bacteria. Perhaps the reaction induced by the hairy-root tissue may have stimulated nonparasitic growth in some cases. These results appear to be in conformity with those of Riker et al. (*26*).

TABLE 1.—*Summary of isolation trials from different kinds of enlargements*

Surface character of enlargement [a]	Isolation trials	Enlargements yielding—		
		Phytomonas rhizogenes	Nonpathogenic bacteria	No bacteria
	Number	*Percent*	*Percent*	*Percent*
Convoluted, with roots	114	60	24	16
Convoluted, without roots	13	39	54	7
Undulated, without roots	25	0	32	68

[a] Characterization of these enlargements is given in the text.

The hairy-root organism was reisolated from hairy-root enlargements induced on nursery apple trees by inoculating the stems below ground. These induced enlargements, ranging in age from 0 to 24 weeks, covered the complete range of symptoms. The same isolation technic was employed as in previous experiments. The results are summarized

in table 2. All stages of the disease yielded the causal organism. Out of 129 trials, the hairy-root organism was recovered in 87 percent, indicating that the causal organism is usually associated with the symptoms of the disease. Nonpathogenic bacteria were recovered in 13 percent of the trials. None of the enlargements was found to be free from bacteria.

TABLE 2.—*Summary of reisolation trials from infectious hairy-root enlargements of different ages, induced by inoculations on nursery apple trees*

Year	Period following inocula- tions	Isolation trials	Enlargements yielding—	
			Phytomonas rhizogenes	Nonpatho- genic bacteria
	Weeks	*Number*	*Percent*	*Percent*
1929	0–4	27	93	7
	5–8	20	60	40
	9–12	8	50	50
1930	0–4	18	100	0
	5–8	14	80	20
	9–12	10	100	0
	13–16	14	100	0
	17–20	12	100	0
	21–24	6	100	0
Total		129	87	13

The roots of infectious hairy root have not been found to contain the hairy-root bacteria. Only negative results were secured from attempts to isolate the organism from the tissues of 37 fleshy hairy roots more than one fourth of an inch long, which had emerged from the basal enlargements. This finding confirms the work of Smith et al. (*36*) and Riker et al. (*26*), who found the bacteria to be present only in the basal enlargements.

Isolation trials from crown gall and wound overgrowth induced by inoculations and wire girdles, respectively, appear to establish the identity of these malformations. A series of reisolation studies some-what less extensive than those conducted on hairy root were made on induced crown gall and wound overgrowth. In 26 trials the crown-gall organism was found constantly associated with the crown-gall symptoms in all stages from 0 to 12 weeks. As no pathogenic bacteria were secured in the 10 trials from wound overgrowths, these malforma-tions apparently are distinct from each other and from hairy root. These results are in accord with those of Riker and Keitt (*30*), Muncie (*17*), Siegler (*33*), Brown (*6*), and others, and, in the writer's opinion, establish the identity of the hairy-root disease.

ENTRANCE OF ORGANISM INTO HOST

MATERIALS AND METHODS

In most of the field studies the Yellow Transparent variety of apple was selected as host plant because it was considered relatively sus-ceptible to infectious hairy root and because it was grown in sufficient numbers for the studies projected. Both first- and second-year trees were utilized. These trees were produced from piece-root grafts made from scions and roots grown in Kansas.

The principal plants used in the greenhouse were Paris daisy, bean, sugar beet, sedum, and apple. In a series of pathogenicity studies

sedum and sugar beet were found to be the best plants tried for greenhouse studies. In these studies an average of 54 inoculations were made for each kind of plant. The following percentages of wounds in the respective hosts showed hairy-root symptoms 2 months after inoculation: Balsam, 36; bean, 58; bryophyllum, 57; Paris daisy (single yellow), 66; Paris daisy (single white), 36; sedum, 100; sugar beet, 100; begonia, 0; and tomato, 0. A similar study of 240 wound inoculations on the Fameuse variety of apple grown in pots showed 52 percent infected.

The bacterial culture chiefly employed was the progeny of a single cell, strain C–1 (fig. 1), of the hairy-root organism, a detailed description of which is given by Wright, Hendrickson, and Riker (*42*). At 3-month intervals this strain was checked for comparative pathogenicity against four sister single-cell strains, C–10, C–11, C–12, and C–13. Three-day-old transfers grown on potato-mannite agar gave abundant growth and were used for inoculation.

The method of inoculation on herbaceous plants was by needle puncture. The method of inoculation on apple grafts, unless otherwise noted, was as follows: On one side of the row a trench was made in the soil to the depth of the union, about 3 inches away from the trees to avoid injuring them. By means of a dibble the soil was removed from around the individual trees, and the stems were wiped free from soil. Drops of a subculture of the bacteria were then applied with a cotton swab to the stem surface, usually in five places spaced about 1 inch apart. With a scalpel, held at an angle, two thrusts were made through the drops of culture deep into the stem. During the 1930 season, strips of adhesive tape were applied over the wounds to reduce chance contamination of the controls from the soil and to keep out insects. Promptly after inoculation the soil was thrown back into the trench and was hilled up several inches so as to bring it about 2 inches above the topmost wound.

WOUNDS AS INFECTION COURTS

The entrance of bacteria into the host has appeared to be a critical stage in the life history of the hairy-root organism; consequently a series of studies was undertaken to clarify this point.

As the bacteria seemed to enter the host plant only through wounds, the necessity for wounds as points of infection was tested in the following preliminary experiments. The hairy-root organism was washed over the surface of 20 Paris daisy plants. Ten of these were wounded with needle punctures in five places each, and the others were held without injury as controls. After 2 months of incubation all the unwounded plants were free from disease, whereas 41 out of 50, or 82 percent of the places wounded, showed hairy-root symptoms. Two repetitions of this experiment gave similar results. An experiment on sugar beets, in which the same number of plants were used and the same number of wounds were made, resulted in the unwounded plants remaining healthy and all 50, or 100 percent, of the wounded plants becoming infected. In two similar experiments on bean, 62 and 34 percent, respectively, of the places wounded became infected, but none of the unwounded plants showed symptoms of disease. In an experiment on the Yellow Transparent variety of apple the bacteria were washed over 20 stems below ground. Ten of these were wounded in five places each with scalpel cuts. Two months later

the unwounded stems were healthy and 28 of the 50 places wounded, or 56 percent, showed symptoms of the disease. These results are in accord with those obtained by Smith et al. (*36*), Riker (*21*), and Muncie (*17*), in work with the crown-gall organism. From these studies it appeared that infection occurred only through wounds. Attention was next directed to the various kinds of wounds that might be encountered.

Infection followed the introduction of the bacteria into the tissue. This was determined by several tests. Drops of a fresh culture of the hairy-root organism from a cotton swab were placed in five different places on the stems of 10 Paris daisy plants. The bacteria were carried into the tissues by thrusting a needle through the bacterial masses and then entirely through the stems several times. After 2 months of incubation, 38 of the 50 places wounded, or 76 percent, showed the disease symptoms. A repetition of this experiment gave disease reactions for 28, or 56 percent, of the places wounded. Similar studies in which the same number of wounds were made on bean, sugar beet, sedum, and apple, gave, respectively, 34, 100, 100, and 48 percent of infections after incubation periods of about 2 months. This type of inoculation technic was very effective in producing infection, and by means of a scalpel instead of a needle it was largely used in the field experiments on apple trees.

Infection followed the placing of bacteria on the surface of wounds. This was determined by a series of tests. In a preliminary experiment a set of 10 Paris daisy plants were wounded by passing a needle through the stems in each of 5 places. The bacteria were then applied to the wound surfaces in the usual manner with a cotton swab. After 2 months of incubation, 36 of the places wounded, or 72 percent, showed symptoms of the disease. As shown later, the results of the infection-court studies, in which this inoculation technic was used, confirm the results of the Paris daisy experiment. These results demonstrate that infection takes place whether the bacteria are applied to the stem before or after the wounds are made and are in accord with those of Riker (*21*) in experiments with the crown-gall organism.

Because of the importance of wounds in bringing about infection, grafting time is an important period for the infection of piece-root-grafted apple trees. Siegler and Piper (*35*) discuss this point. Whether the bacteria commonly do gain entrance at this time is of such importance that it is discussed in a separate paper, on studies of the seasonal development of the disease, by Riker and Hildebrand (*28*). The reactions at the graft union present so many complications that certain phases of this difficult problem have been simplified in the present work through studies of wounds on the scions of actively growing trees.

INFLUENCE OF TYPE OF WOUND

In a series of trials the type of wound apparently made no difference in the kind of overgrowths but did influence somewhat their rate of development and the percentage of wounds that became infected. A preliminary study on a comparison of needle-puncture and scalpel-cut wounds was made on underground stems of first-year apple trees to test their influence in producing infection. Five needle-puncture wounds were made in each of 10 different trees. An equal

number of scalpel cuts were made in 10 trees. The procedure was to push the scalpel or needle deep into the stem, and to smear the hairy-root bacteria promptly over the wound with a cotton swab. Host injury was obviously greater from the scalpel cuts, and after an incubation period of 2 months infection seemed to be more pronounced from the scalpel injuries. Subsequently however, this difference gradually diminished. In this experiment all the injured trees became infected. The percentages of infection resulting from needle-puncture and scalpel-cut wounds were 38 and 62, respectively. In the percentage of wounds infected there was a discrepancy between the two types of wounds, and this required further consideration.

A more extensive wound-type study was made to determine whether the hairy-root bacteria could enter apple stems through different types of injuries. Three types of wounds were employed, as follows: (1) Scalpel cut, which was made by pushing a scalpel deep into the stem at an angle; (2) bruise, which was made to resemble cultivation injury by striking the stem with a hammer; and (3) needle puncture, which was made by thrusting a needle deep into the center of the stem. The effects of these injuries were studied on first- and second-year trees. Each type of wound was made in 5 places on the stem below ground on 13 trees of both ages. In each case the hairy-root bacteria were promptly applied to the surface of the wounds on 10 trees. The wounds on 3 trees were left untreated, as controls. Ten weeks later the results were taken. Because of the similarity of reactions on the first- and second-year trees, they are summarized together. For the respective types of injury the percentages of wounds becoming infected were as follows: Scalpel cut, 71; bruise, 66; and needle puncture, 41. Of the control wounds 5 percent of both the scalpel and bruise injuries became infected. The infection of the control wounds might be attributed to some soil vector, such as insects, which had free access to the wounded places. From this study it is apparent that all the different types of injuries served as infection courts. Moreover, the symptoms produced by the infection of the different wounds were practically identical except for those in the needle-puncture injuries, which differed from the others in having (1) a slightly longer incubation period, (2) a somewhat lower percentage of infections, and (3) smaller reactions.

A repetition during 1931 of the experiment testing the three types of wounds (scalpel cut, bruise, and needle puncture) on second-year trees gave results similar to those just described. Strips of adhesive tape were placed over all the places wounded as a protection against the possible interference of soil fauna. Scalpel-cut and bruise wounds again were the most effective infection courts for the hairy-root bacteria, as 82 and 66 percent, respectively, of these wounds became infected. Thirty-six percent of the needle-puncture wounds became infected. All the control wounds were negative. The slightly longer incubation periods for smaller wounds suggested the need for further study of the size of wound in relation to the development of the disease.

Extremely shallow wounds were found to be poor infection courts. In 1930 two types of wounds were tested, surface and scalpel cut. The surface injury consisted of a very shallow scraping of the stem to expose the outermost cortex layers to the bacteria. The scalpel cuts were made as before. Each type of injury was made in 5 different

places on each of 40 trees. The wounds on 30 of the trees were inoculated with the bacteria, those on 10 trees being left as controls. All the wounds were covered with adhesive tape. After an incubation period of 10 weeks, 65 percent of the scalpel wounds and only 3 percent of the surface wounds had become infected. An identical experiment repeated in 1931 gave confirmatory results, as 82 and 4 percent, respectively, of the two types of injuries became infected. These results show that shallow injuries to the stems of young apple trees are relatively poor infection courts and that, within limits, the depth as well as the size of wounds is a factor in the amount of hairy-root infection.

LENGTH OF TIME WOUNDS REMAIN OPEN TO INFECTION

The length of time that wounds remain open to infection was next considered. Preliminary studies were made on Paris daisy, sedum, and bean plants in the greenhouse. The temperature of the greenhouse was approximately 22° C. and the relative humidity about 70 percent. In the first series 5 needle-puncture wounds were made in the stems of 22 Paris daisy plants above, at, and below the ground level. At intervals of 1 hour and 1, 2, 3, 4, 5, 6, 7, 8, 16, and 32 days after wounding, a water suspension of a culture of the hairy-root bacteria was applied to the wounds of two plants with a cotton swab. The results were taken after an incubation period of 2 months. It was found that, in this trial in which the plants were kept on an open greenhouse bench, wounds older than 3 days did not become infected. This indicated that wounds 4 days old had formed a barrier sufficient to keep out the organism. Repetitions of the experiment on Paris daisy, bean, and sedum showed that for 3, 2, and 4 days, respectively, the wounds remained open for infection under greenhouse conditions.

These studies were then extended to include the effect of humidity on the time that infection courts remain open in Paris daisy and bean plants. The same time intervals and number of wounds were employed as in the previous tests. Exposure for 2 days before wounding to a relative humidity of approximately 90 percent at the usual greenhouse temperature of about 22° C. caused the wounds to stay open longer for infection. The period was extended to 5 days for both Paris daisy and bean. In a similar experiment, with the same preliminary treatment, on Paris daisy plants held at the same humidity for 9 days after wounding, the period that wounds remained open for infection was increased to 6 days. These results show that wounds on plants in the greenhouse are ordinarily open to infection for only a few days after they have been made and that raising the relative humidity lengthens this period.

The length of time that wounds on apple remained open for infection was approximately 2 days. Field studies on actively growing apple trees in the nursery were made during the seasons of 1929 and 1930. In 1929 three series of infection-court trials were made, starting, respectively, on May 9, June 7, and July 3. In each series, on the day of starting, 5 wounds were made on the underground stems of each of 4 first-year trees for each interval. The intervals tested were 1 hour and 1, 2, 4, 8, 16, 32, and 64 days. Four wounded trees were kept as controls. At the stated time after wounding, a fresh culture of the hairy-root bacteria was applied to the wounds with a

cotton swab. At the close of the season results showed that the wounds on these trees remained open infection courts for no longer than 2 days. These data corresponded closely with those for greenhouse plants. The exact time periods for May, June, and July were, respectively, 2, 2, and 1 days. In this experiment the time of year appeared to influence the results slightly. An identical experiment was conducted on second-year trees with almost the same results. However, in this case, in the June series, 2, or 10 percent, of the control wounds and 5, or 7 percent, of the wounds inoculated later than 4 days after wounding became infected. These results caused the writer to suspect the interference of insects.

Similar studies in 1930 consisted of five series, starting, respectively, on May 12, June 1, July 1, August 1, and September 1. The procedure of the previous season was followed throughout except that the unnecessary 32- and 64-day intervals were discarded. Both first- and second-year trees were used. To prevent possible interference from hairy-root bacteria or insects that might be present in the soil, adhesive tape was placed over the wounds promptly after they were made. The results (table 3) are in accord with those of the season before on the first-year trees, and fix the time that wounds ordinarily remained open for infection at approximately 4 days for the month of May and 2 days for the other months. Since the application of the adhesive tape over all the wounds the second season eliminated chance infections other than those shown in the table, it appears reasonable to assume that the tape was a barrier to some wound-producing element in the soil environment. The fact that the weather was more favorable for host growth during May 1929 than during May 1930 would seem to account for the discrepancy of 2 days in the length of time the wounds remained open for infection. Figure 1, *B*, illustrates the typical hairy-root infections for the May series, when the plants were 24 weeks old.

TABLE 3.—*Length of time at different periods in the growing season of 1930 that wounds on 1- and 2-year-old apple trees remained open infection courts for the hairy-root organism* [a]

Period between wounding and applying bacteria	Wounds on trees of indicated age infected during—									
	May		June		July		August		September	
	1 year	2 years	1 year	2 years	1 year	2 years	1 year	2 years	1 year	2 years
	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
1 hour	15	18	17	16	16	15	20	18	12	12
1 day	16	16	8	16	12	13	15	9	8	9
2 days	16	16	4	2	11	10	12	10	4	7
4 days	9	9	0	0	0	1	0	0	0	0
8 days	0	0	0	0	0	0	0	0	0	0
16 days	0	0	0	0	0	0	0	0	0	0

[a] Wounds were made on May 12, June 1, July 1, Aug. 1, and Sept. 1. 20 wounds were made in each trial. Observations were made on Nov. 10.

The studies on the length of time that wounds remain open infection courts both furnish support to and receive support from similar studies on whether callus may be ordinarily an infection court. Callus was found ordinarily to be a barrier against the invasion of the hairy-root organism. Riker and Keitt (*30*) earlier reported that callus formed on apple grafts was not commonly an open infection
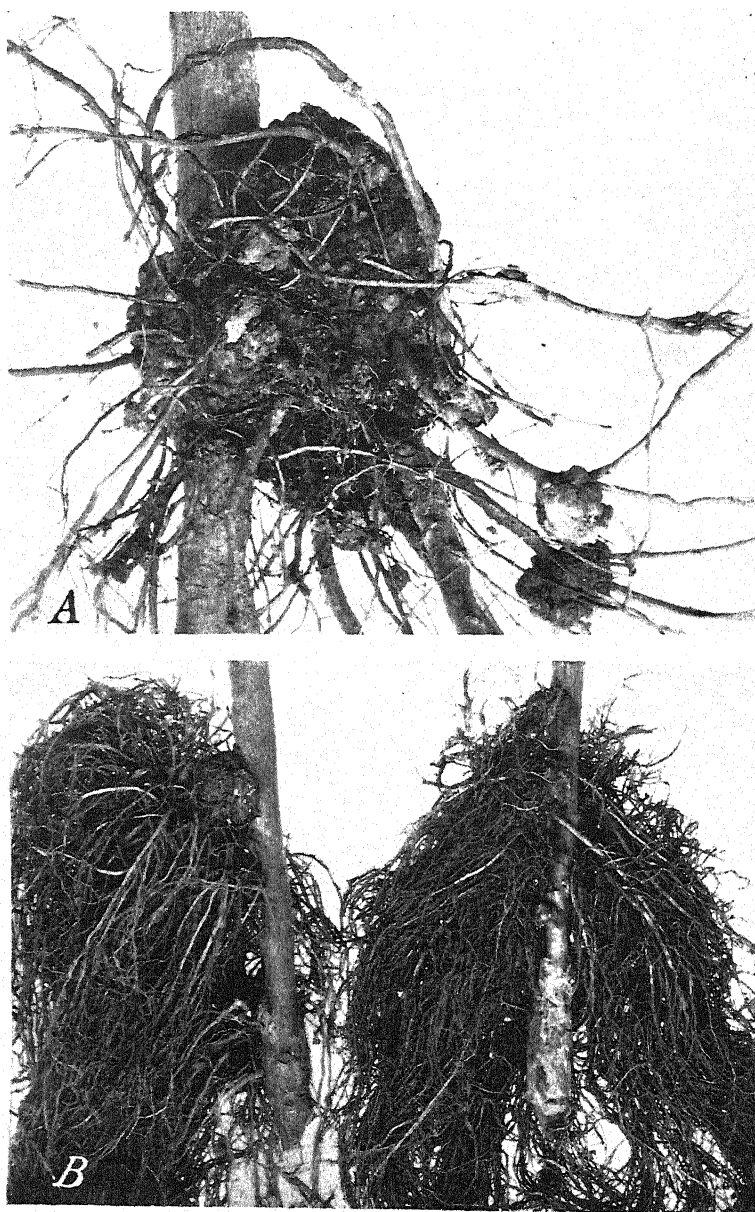
FIGURE 1.—*A*, Hairy-root overgrowths on the lateral roots of a tree infected on the main stem. These lateral overgrowths, which yielded the hairy-root bacteria, presumably followed insect wounds. $\times$ ⅔. *B*, Hairy-root infections 24 weeks after inoculation. The bacteria were applied to the specimen on the left 1 hour after the wounds were made and to the specimen on the right 1 day after the wounds were made. $\times$ ½.

court for the crown-gall organism.   Siegler (*34*) stated that "the graft union after callus has formed is an infection court to a limited extent for the apple organism."   Siegler and Piper (*35*) reported a confirmation of Siegler's earlier results, stating that "the grafts are m st susceptible at the time they are made and become increasingly resistant with the progression of callus formation."

<div align="center">CALLUS AS A BARRIER TO INFECTION</div>

The infection of callus was studied on apple trees in the nursery. Callus formation was stimulated by wounding the stem, and its reaction toward the application of the bacteria was tested in the soil under natural conditions.   Callus of different ages was stimulated by making five wounds below ground on each of eight trees weekly for 10 weeks.   On the last date of wounding the bacteria were applied to the wounded surfaces of one half the number of trees.   Examination 2 months after inoculation revealed that only those trees wounded on the date of inoculation had become infected.   Of these, 11 out of 20, or 55 percent, showed the disease symptoms.   The entry of the bacteria in this case had been accomplished before callus had had time to form.   Comparable results were obtained in a study by Riker, Hildebrand, and Ivanoff (*29*), similar to that just described except that the callus was stimulated above ground in glass cylinders under approximately aseptic conditions.   Similar studies, except for minor changes, were made in 1930.   The time, originally 10 weeks, was extended to 12 weeks.   Strips of adhesive tape were applied over the wounds to exclude possible soil bacteria and insects.   Otherwise the procedure was the same.   Again wound callus more than 1 week old did not become infected.   Nine out of the 20 wounds inoculated on the day of wounding, or 45 percent, showed symptoms of the disease before callus had had time to form.   The results of the season before were verified.

Further studies of callus as an infection court were made in 1931. In one experiment several turns of wire were wrapped around the underground stems of eight trees at weekly intervals for 12 weeks. One week later for each interval, the wrapped portions of the stems of four of the trees were smeared with the bacteria.   The other four trees were left untreated, as controls.   Observations were made 9 weeks after the application of the bacteria.   Although disease symptoms appeared in callus developments of all 12 ages, in 10 percent of the inoculated trees and in 8 percent of the control trees, this study seemed to demonstrate that callus is not commonly an infection court.   The fact that a white grub was observed feeding on one of the overgrowths which later showed infection indicates that the chance infections encountered may have been due to insect injuries.

In another experiment callus was stimulated by making a slanting upward cut with a scalpel into the underground stems.   To prevent reunion of the several tissues a strip of adhesive tape was inserted in each cut.   In all other particulars this experiment was identical with the preceding one.   When the observations were made, it was found that 12 percent of the inoculated trees were infected and that the control trees were not infected.   These results appear to substantiate those just reported in showing that unwounded callus does not ordinarily serve as an infection court.[4]

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[4] Illustrations of these callus developments will be found in a paper by Riker and Hildebrand (*28*).

Since callus is very easily injured, a study was made of the suscepti-
bility to hairy-root infection of callus that had received small wounds.
Shallow wounding did not commonly, if at all, permit the entry of the
hairy-root organism. Using the technic previously described, the
writer stimulated callus by wounding under three conditions: (1) On
underground stems exposed to the soil; (2) on underground stems
protected by adhesive tape; and (3) on aerial stems in glass cylinders
(29) protected from outside contamination. At 4 biweekly intervals 5
wounds were made in each of 2 plants for each of the 3 conditions
named above. One week after the last wound was made the callus
developments of the various ages were lightly pricked and scratched
so as to limit the injury to the outer few layers of callus cells. The
bacteria were then smeared over the injured surfaces. After an
incubation period of 8 weeks only one of the wounds, and that in a
1-week-old callus, was observed to be infected. In this case it
appeared that, after the injury was made, insufficient callus remained
over the susceptible host tissues to be a barrier to the organism.
Parallel to this an equal number of wound inoculations were made
which resulted in the development of the disease. Repetition of
one part of this experiment, namely, that on the underground stems
protected by adhesive tape, gave the same results the next season.
These results indicate that shallow wounds in callus do not ordinarily
bring about infection, which is in accord with the results obtained
when shallow wounds were made in apple stems.

INFLUENCE OF SOIL INSECTS

Wounds produced by insects should be considered as a possible
factor in infection by the hairy-root organism in the nursery. Root-
chewing arthropods have been found by Banfield (4) associated with
the occurrence of crown gall on raspberry. He showed that healthy
raspberry plants grown in soil which had been inoculated with the
crown-gall organism but which was free from insects became diseased
only when white grubs were introduced into the controlled environ-
ment. Consequently it seemed desirable to examine the possible
relation of insects to hairy-root infection.

Root-feeding insects have been observed during three growing
seasons in the soil about nursery apple trees. Preliminary to a more
detailed study (28) of seasonal development of diseases, including
hairy root, a survey was made of the insects commonly present around
the graft unions in the soil. From the survey it was determined
that root-feeding insects, especially white grubs (Phyllophaga) and
wireworms (Elateridae), were commonly present in the nursery
soil during the growing season, from May to October.

Insects eating both hairy-root and healthy tissue have frequently
been seen during the observations of diseased and healthy trees.
These observations were made in the course of studies which required
the examination of several hundred young apple trees each week.
On several occasions during the growing season, from May to October,
white grubs were observed feeding upon hairy-root overgrowth tissue
and other underground parts, such as stem tissue, root tissue, and
both infectious and noninfectious hairy roots. Observations in 1931
served to support the findings of the two previous seasons as to the
activity of this insect and pointed to it as an important agent in

producing wounds that might lead to the hairy-root disease if the causal bacteria were present.

Injuries similar to those produced by white grubs in Kansas have been observed in Wisconsin, Iowa, Missouri, Nebraska, and Oklahoma. Wireworms also were occasionally observed burrowing into callus and overgrowth tissues, especially in the earlier stages. Small fungus-gnat larvae (Mycetophilidae) were found to frequent some of the enlargements in the crevices and sometimes the tissues. On three occasions white ants were found making trenches in the main root or in the large branch roots, sometimes for the greater portion of their length. Twice microscopic examination of the surface of five young overgrowths revealed the presence of nematodes. These are but some of the instances of interference by soil fauna with the underground plant parts as observed in the nursery during the growing season.

Over a period of 4 years from one to several specimens of infected lateral roots were observed in positions that could have been reached only by insects. Riker and Hildebrand (28) in their studies found from 0 to 0.6 percent of the lateral roots infected. Hairy-root bacteria were isolated from the small overgrowths and from the main overgrowth in the specimen illustrated in figure 1, *A*, proving their infectious nature.

The development of new infections during both the first and the second growing season in Kansas was most easily explained as due to the agency of insects. As stated earlier, wounds appear to be necessary for the entrance of the bacteria. To account for the occurrence of new infections over so long a period, some wound-producing agency must, therefore, have been almost constantly present in the soil around the graft unions. Two examples may be cited. In a block of 240 trees, 39 percent were found infected at the end of the first growing season and 59 percent at the close of the second growing season. A summary of the studies over a period of 4 years, in which about 7,000 trees were examined, showed at the close of the second season an increase in disease development of 13 percent over the first season. Further evidence on this point will be found in the paper on seasonal development by Riker and Hildebrand (28).

Several experiments were suggested by the observations on insects in relation to hairy root. Isolations of the hairy-root bacteria were made from white grubs that had been feeding on diseased tissue. Out of 38 isolation trials from the alimentary tracts of white grubs in 1930, only one culture of the bacteria was obtained. The method of isolation consisted in disinfecting the exterior of the insect by immersion for 10 seconds in mercuric chloride, 1 : 1,000, and removing the digestive tract, which was transferred directly to 100 cc of sterile distilled water. The vessel containing the water and digestive tract, after being shaken, was allowed to stand for 30 minutes, when five 1-cc portions were plated on bile agar (19). Out of five isolation trials in 1931 from the mouth parts of white grubs which had been eating young hairy-root overgrowth tissue when collected the day before and on which no surface disinfectant was employed, two cultures of the hairy-root bacteria were obtained. Eleven isolation trials from the alimentary tracts of as many white grubs, when no disinfectant was employed, were all negative. These studies are of a limited nature. However, the fact that some insects which habit-

ually feed upon underground plant parts may carry the hairy-root bacteria even for a short time is of significance in the life history of the hairy-root organism.

Isolations from other insects gave negative results. Regardless of the significance that may be attached to these isolation studies, the fact remains that wounds produced are potential infection courts for the bacteria.

Insect repellents—paradichlorobenzene (*9*) and mercuric chloride (*7, 10*)—were employed in an effort to reduce the amount of insect injury and consequently of hairy-root infection on first-year trees in the nursery. Although 50 and 35 percent reduction in hairy root was secured, the data are omitted because they are insufficient for definite conclusions.

Insect barriers made of treated cloth were tested. Cloth of un-bleached muslin, 50 meshes to the inch, was washed to remove the filler, and dried. Strips 4 inches wide were folded and sewed to make a sack approximately 1 inch in diameter and 4 inches long. The sacks were soaked for 30 minutes in a preservative solution of 29 parts (by weight) of copper oleate dissolved in 71 parts of gasoline. This treatment has been successfully used in the preservation of cotton fish nets in both sea and lake water (*38*). The sacks were slipped over the graft unions and fastened at the base by folding and securely wrapping with a strip of adhesive tape 4 inches long by one half of an inch wide. Autoclaved soil was introduced from the top in suffi-cient amount to keep the cloth from contact with the union. The top of the sack was then folded and wrapped in the same manner as the bottom. Grafts wrapped with string and made from the same supply of scions and roots were used in this experiment. Five hundred grafts were fitted with sacks. An equal number without sacks were planted alongside. The results taken at the close of the season showed about 6 percent of the sacked grafts and 24 percent of the controls infected. The cloth resisted decay until August, when an occasional sack had begun to show disintegration. Along with this treatment there was a growth-retarding action from the use of the sacks of about 4 inches to the tree. Moreover, about 16 percent more of the sacked trees than of the controls died during the summer. Although the reduced amount of disease for the sacked grafts may have been partly due to one or more causes besides the cloth barrier, the evidence ap-peared to warrant a repetition of this experiment. The following season a similar study gave more reliable results. For the sacked and control trees there were, respectively, 13.9 and 26.0 percent of disease, 75 and 76 percent of stand, and 32.6 and 34.4 inches of aver-age height. With so little difference in stand and height, the difference in percentage of disease appears important. The possibility of anti-septic action by copper oleate upon the hairy-root bacteria was tested but was not found to be significant. These experiments gave added strength to the idea of insect interference.

INFLUENCE OF CONDITION OF HOST

The entrance of the hairy-root bacteria into nursery apple trees was studied in relation to the susceptibility of the host as influenced by (1) the time of the growing season, (2) the age of the tree, (3) the size of the tree, (4) the variety of the tree, and (5) the previous infection of the tree.

The period of the growing season seemed immaterial so far as entrance of the organism was concerned. This was determined by making inoculations at weekly intervals throughout three growing seasons. Each week 10 trees were inoculated in the usual way by introducing the bacteria through scalpel cuts in the stems in five places. The percentage of trees that became infected was used as the measure of the entry of the bacteria. The results for the three seasons are given in table 4. Except at sporadic intervals during the growing seasons studied, the trees permitting entry of the bacteria approached 100 percent, the averages for 1929, 1930, and 1931 being 97, 96, and 98 percent. The apparent exceptions seem to be within the range of experimental error. The rapid falling off in the percentage of disease in September 1929 was probably due to the early dormancy of the trees. Of the inoculations made on September 4 and September 11, 50 and 0 percent, respectively, had become infected by the time of the last observation in November. This idea was verified when in May 1930 it was found that all the trees which had apparently become dormant the September before showed infection. Consequently, 100 percent infection is shown in table 4. The bacteria had entered and overwintered in the wounds, ready to produce infection the following spring. Corresponding inoculations in September 1930 and September 1931 produced disease symptoms in November. It appears, therefore, that bacteria will enter the host during any part of the growing season and will produce infection if the trees have not become dormant.

TABLE 4.—*Wounded apple trees infected by hairy-root bacteria at different periods during 1929, 1930, and 1931*

Date	Trees infected [a]	Date	Trees infected [a]	Date	Trees infected [a]
1929	*Percent*	1930	*Percent*	1931	*Percent*
May 15	90	May 12	100	May 11	100
May 22	100	May 19	100	May 18	100
May 29	100	May 26	100	May 25	100
June 5	100	June 2	100	June 1	100
June 12	100	June 9	100	June 8	90
June 19	90	June 16	50	June 15	100
June 26	100	June 23	100	June 22	100
July 3	100	June 30	100	June 29	90
July 10	100	July 7	100	July 6	100
July 17	90	July 14	100	July 13	100
July 24	100	July 21	100	July 20	100
July 31	100	July 28	100	July 27	100
Aug. 7	100	Aug. 4	90	Aug. 3	100
Aug. 14		Aug. 11	90	Aug. 10	100
Aug. 21	100	Aug. 18	100	Aug. 17	90
Aug. 28	80	Aug. 25	100	Aug. 24	90
Sept. 4	[b] 100	Sept. 1	100	Aug. 31	100
Sept. 11	[b] 100	Sept. 8	100	Sept. 7	100
				Sept. 14	100

[a] 10 trees were inoculated each week.
[b] For explanation see paragraph above table.

The age of the nursery apple tree, in this region where trees are grown only 2 years, did not seem to influence susceptibility to infection by the hairy-root bacteria. Inoculations made in 1929 on first- and second-year trees showed 85 and 93 percent of infection, respectively, and inoculations made in 1930 and 1931 showed 97 percent of infection on both first- and second-year trees. An average of 40 trees, each wounded in five places, was used in each part of the test.

All sizes of growing apple trees were found infected in the same field under natural conditions. Each year, at the end of the growing season, representative first-year trees were selected, examined for disease development, and measured for height. A graphic presentation (fig. 2) of the data accumulated during the 1929 season serves to
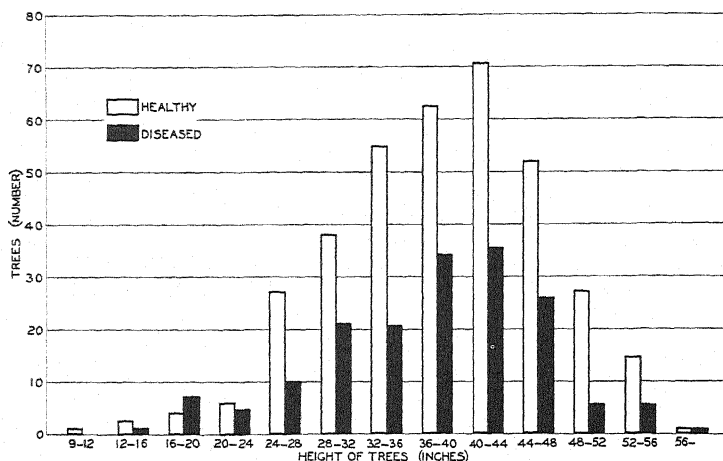
FIGURE 2.—Distribution in 1929 of healthy and hairy-root diseased 1-year-old apple trees in groups based on height of trees in inches.

bring out the distribution of the diseased and healthy trees with respect to the different size groups arbitrarily chosen. For graphing, all the trees were placed in size groups differing from each other by intervals of 4 inches, e.g., (1) less than 12 inches, (2) 12 to 16 inches, (3) 16 to 20 inches, etc. These data showed a fairly even distribution of both diseased and healthy trees over the range of size groups. A similar study on second-year trees (fig. 3) gave similar results. Since all
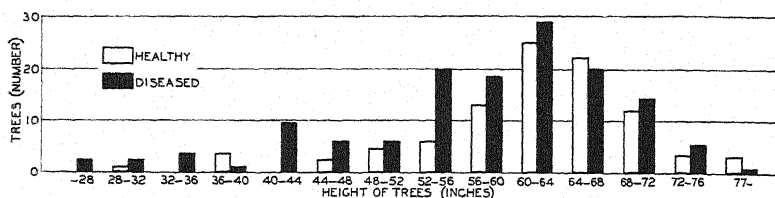
FIGURE 3.—Distribution in 1930 of healthy and hairy-root diseased 2-year-old apple trees in groups based on height of trees in inches.

sizes of trees may be found infected in the field it seems logical to conclude that size is not an important factor in susceptibility of trees to infection by the hairy-root organism. Further evidence in support of this conclusion was derived from an experiment in which 311 trees as they came in the row were inoculated; subsequently, 95 percent were found to have become infected. When compared as to size it was found that most of the 5 percent which resisted infection fell into the more numerous middle-sized groups.

Varietal susceptibility to infection by the hairy-root organism differed widely among the varieties of trees studied. That not all

varieties of apple are equally susceptible to the hairy-root disease has been recognized by different workers, including Hedgcock (*12*), who reported as the result of a country-wide survey that the Ben Davis, Wolf River, and Northern Spy varieties especially were attacked in the nurseries. The present study was made on the relative susceptibility of the varieties of apple trees commonly grown at Topeka, Kans. Late in May 50 inoculations were made in each of 29 varieties in 1930 and in each of 37 varieties in 1931. Twenty-seven of the same varieties appeared in the tests both years. The varietal names were checked with "Standardized Plant Names" (*2*), the local names being retained when the variety did not appear in the standard list. Two names are given for some of the varieties. After 2 months of incubation the overgrowths were cut off even with the stem surface, wrapped in moist paper, and taken to the laboratory. The following points were recorded for each variety: (1) The number of inoculations giving positive reactions, (2) the volume displacement of hairy-root tissue, (3) the wet weight of hairy-root tissue, and (4) the dry weight of hairy-root tissue.

On the basis of the percentage of wounds infected, the different varieties ranged in susceptibility from 12 to 100 percent in 1930 and from 22 to 100 percent in 1931 (table 5). Three of the 27 varieties appearing both seasons (Fameuse, Florence (crab), and Livland Raspberry) gave the same percentage of positive inoculations; three others (Hopa (crab), McIntosh, and Wealthy) gave a lower percentage of positive inoculations the second season; the remaining 20 varieties showed an increase in the percentage of positive inoculations the second season. For example, the Yellow Transparent variety had a susceptibility to infection of 48 percent in 1930, and 82 percent in 1931. On the basis of growth, the varieties showed some difference with respect to one another and considerable difference with respect to the season. The average amount of hairy-root growth for each positive inoculation was calculated on the three bases already given.

Since the three different measures of hairy-root development showed such a close correlation, only the first one need be discussed. The volume growth in cubic centimeters of the different varieties ranged from 0.31 to 1.71 in 1930 and from 1.00 to 4.04 in 1931. Without exception, in the 27 varieties appearing both seasons a considerably larger growth of diseased tissue occurred the second season. When the varieties were grouped the average growth of diseased tissue in 1931 was approximately 300 percent of that of 1930. These studies indicate that varietal differences in susceptibility to the hairy-root organism are important factors in the infection and development of hairy root.

In 1929 50 second-year trees which had become diseased the first season were wounded by deep punctures made with a pointed blade above, below, and at either side of the union, at least 1 cm from the enlargements; 25 other diseased trees were kept unwounded as controls. A group of 25 healthy trees were wounded about the union in the manner described above; 50 others were left unwounded as controls. The soil in which the trees were growing was infested with the hairy-root bacteria. Of the diseased trees, 24 percent of those wounded and 12 percent of those left unwounded showed new infections at the end of the year; of the healthy trees, 28 percent of the wounded and 14 percent of the unwounded became infected. This

study revealed the fact that new infections occurred whether the trees were diseased or healthy. Moreover a number of new infections occurred in the same series where no wounds were made, the majority of these infections appearing at the union or above it on the scion. Numerous observations in the field confirm these findings. Apparently previous infection of nursery apple trees does not produce an immunity to later infection. This is in accord with the results obtained by Smith et al. (*36*), Brown (*5*), and Riker (*22*), in their work with crown gall.

TABLE 5.—*Results of tests made during 1930 and 1931 in Kansas on the relative susceptibility of 38 varieties of nursery apple trees to infection by the hairy-root organism* [a]

Variety	Inoculations positive		Average growth of reactions measured by—					
			Displacement		Wet weight		Dry weight	
	1930	1931	1930	1931	1930	1931	1930	1931
	Per-cent	*Per-cent*	*Cc*	*Cc*	*Grams*	*Grams*	*Grams*	*Grams*
Anoka	34	76	0.60	1.58	0.51	1.32	0.12	0.27
Arkansas [b]	20	56	.65	1.21	.50	1.06	.10	.18
Baldwin	42	86	.31	1.16	.26	1.05	.05	.21
Beauty (crab)		34		1.30		1.03		.19
Ben Davis	72	88	.39	1.89	.38	1.50	.07	.27
Cortland	100		.88		.80		.19	
Delicious	62	94	.45	1.70	.36	1.57	.07	.37
Dolgo (crab)		98		3.51		3.08		.66
Early Cooper	98	100	1.71	3.78	1.40	3.43	.26	.61
Early Harvest	26	68	.69	1.56	.59	1.33	.15	.27
Fameuse [c]	94	94	1.49	3.77	1.41	3.46	.22	.60
Florence (crab)	60	60	1.13	3.33	1.02	3.05	.18	.51
Gano I [d]	30	100	.50	3.04	.45	2.77	.07	.62
Gano II		92		1.72		1.63		.29
Grimes Golden	38	84	.80	1.38	.67	1.24	.13	.27
Hopa (crab)	38	22	.42	1.45	.34	1.07	.08	.20
Hyslop (crab)	98	100	1.39	3.86	1.28	3.72	.25	.76
Jonathan	56	92	.61	2.59	.55	2.39	.10	.47
Livland Raspberry [e]	100	100	1.31	2.14	1.19	1.87	.21	.40
McIntosh	38	36	.60	2.12	.57	2.08	.14	.41
Minkler		70		1.08		1.00		.19
Maiden Blush	86	96	.89	3.17	.78	2.77	.15	.52
Northwestern Greening		90		2.69		2.35		.37
Oldenburg [f]		96		2.73		2.46		.41
Rambo	68		.36		.30		.08	
Red Astrachan	88	98	.60	1.53	.56	1.23	.08	.22
Red June		98		2.96		2.58		.61
Rome Beauty	20	30	.44	1.47	.40	.95	.07	.23
Stayman Winesap	12	86	.33	2.63	.31	2.44	.05	.44
Tolman Sweet	54	84	.46	1.88	.39	1.71	.09	.34
Turley		74		1.65		1.28		.58
Wealthy	74	72	.51	2.22	.49	1.96	.10	.39
Whitney (crab)	14	68	.57	2.44	.46	2.07	.09	.48
Willowtwig		98		2.61		2.31		.39
Wilson June		96		4.04		3.37		.67
Winesap	24	90	.42	2.24	.37	2.03	.07	.44
Winter Banana	60	86	.47	1.00	.40	.87	.08	.15
Yellow Transparent	48	82	.62	2.02	.52	1.83	.11	.37
York Imperial	16	66	.44	1.18	.39	.94	.10	.21
Average [g]	52	78	.69	2.16	.61	1.91	.12	.37

[a] 50 inoculations were made on each variety.
[b] Mammoth Black Twig.
[c] Snow.
[d] Black Ben.
[e] Livland Raspberry.
[f] Duchess.
[g] These averages represent the 27 varieties occurring both seasons.

LOCATION OF ORGANISM WITHIN HOST

The location of the hairy-root organism in the host tissues was another phase of the life history studied. Studies on the location of the crown-gall organism in the tissues of several hosts were made by Riker (*21*), Robinson and Walkden (*32*), and Ivanoff and Riker (*13*). Because of its similarity to the hairy-root organism, the studies on the crown-gall organism suggested methods of approach for this work.

The hosts selected for this study were Paris daisy and apple. Inoculations were made only on young stems of vigorous plants, from 1 to 4 inches below the apex. The method of inoculation was by needle puncture through a drop of a suspension of the bacteria placed on the surface of the stem. Control punctures were made without the bacteria. Inoculated and uninoculated stem specimens were prepared for study, at intervals of 1 hour, 1, 2, 3, 4, 8, and 16 days after treatment. This material was examined both in the fresh state and when embedded in paraffin after being fixed in formalin-acetic-alcohol. Parallel series were prepared in which the inoculum consisted of hairy-root bacteria mixed with *Phytomonas insidiosa* (McCulloch) Bergey et al., a Gram-positive organism, and hairy-root bacteria mixed with india ink. These mixtures, devised to aid in the location of the bacteria in the tissues, are similar to those used by Ivanoff and Riker (*13*). Of a large number of staining combinations tried, including the use of Gram's stain in studying the mixture of organisms, the staining procedure found most satisfactory was to immerse the preparation for about 1 second in dilute safranine, 1:1,000, followed by the light-green counterstain.

Relations of the hairy-root organism with the host are apparently first begun in the liquid released by the wounds. When wounds were made in the stems of Paris daisy and apple, there was a water-soaking of the neighboring tissues similar to that described by Riker (*21*) in tomato. These juices, liberated from the cells by the wound, flooded the neighboring intercellular spaces for a short distance from the wound cavity. The bacteria were observed in the wound cavity and for some distance between the cells in the surrounding host tissues. The bacteria, operating under a complexity of forces, as suggested by Ivanoff and Riker (*13*), become distributed in the wound juices that ordinarily fill the neighboring intercellular spaces.

In the fresh material the bacteria appeared to be located in the intercellular spaces in both Paris daisy and apple stems. For example, 8 days after inoculation a comparison of both Paris daisy and apple tissues which had received control punctures was made. The crushed tissues bordering the wound cavity in both inoculated and uninoculated stems were discolored. The inoculated stems had an intercellular discoloration spreading out into the tissues for a short distance from the path of the needle. It varied from a pale yellow to a brown and was especially marked in the tissues outside the cambium. None of the uninoculated tissues showed this discoloration. From the limited studies made the position of the discoloration, whether in the walls or in the material between the cells or in both, could not be determined with finality. However, in the section examined, it seemed to be present in both places in varying amounts. The extent of the discoloration between the cells from the edge of the wound seemed to correspond rather closely to the area which appeared water-soaked when the

wound was made, roughly a distance of 10 to 20 cells. The fact that outside the path of the needle only the inoculated tissue became discolored indicated that this condition was caused by the presence of the bacteria.

The fixed Paris daisy material, whether stained or unstained, was found to contain the bacteria in the spaces between the cells. Sections were cut from 10μ to 20μ in thickness. After inoculation the unstained tissues showed discolorations similar to those of the fresh material. A yellowing of the regions apparently invaded by the bacteria became evident 4 days after inoculation; 8 days after inoculation the color became deeper, almost brown. Staining dyes showed a strong affinity for these regions, so that the bacteria, if present, were obscured. In some sections the use of india ink or Gram-positive bacteria made the invaded intercellular spaces more distinct, although their position and extent seemed to be the same. It was generally found that the discolored regions in 8-day-old preparations took the dyes very readily, making the bacteria difficult to see. Starting from the wound, search was made for the bacteria. Only cells that had been injured were observed to contain bacteria within them (fig. 4, A). The bacteria could be traced for short distances into the intercellular spaces. In the pith of Paris daisy there are comparatively large intercellular spaces. Photomicrographs of a cross section and longitudinal section of stems 8 days after inoculation showed deeply stained material in these intercellular spaces (fig. 4, B and C). With high magnification the bacteria are visible in such locations (fig. 4, D).

Studies of the apple stems involved preparations similar to those on Paris daisy, and showed the bacteria to be similarly situated. The extent of migration of the bacteria into the tissues outside the xylem from the path of the wound seemed even more limited than for Paris daisy. Moreover the bacteria were observed to stimulate hyperplasia only in the tissues outside the xylem.

The presence of the organisms between the cells of the tissues bordering the wound stimulated a multiplication of the cells in those localities. This change appeared to begin about 8 days after inoculation. All the tissues were examined less than 16 days after inoculation. Cross sections of stems made 8 to 16 days after inoculation revealed the presence of circular areas of hyperplasia (fig. 4, E and F) outside the xylem. Variable in number and from one to several cells in radius, these areas were in close proximity to the wound borders and appeared to be about the intercellular spaces containing the bacteria. For a distance of from 1- to 10-cell diameters away from the center of such localities the cells were stimulated to division in a tangential plane. This was the condition 16 days after inoculation. Bacteria have been observed usually at the centers of such areas, which when stained stand out distinctly in strong contrast to the surrounding tissues. The development of hairy root after the formation of the hyperplastic areas is an important phase of the disease requiring further investigation.

In the injured xylem vessels the organisms appeared for a short distance from the wounds in both cross and longitudinal sections of apple and Paris daisy stems. In some longitudinal stem sections of Paris daisy it was common for the bacteria to be present in the vessels to the end of the section. Stem cross sections above and below the wounded area showed the bacteria to be present within the vessels
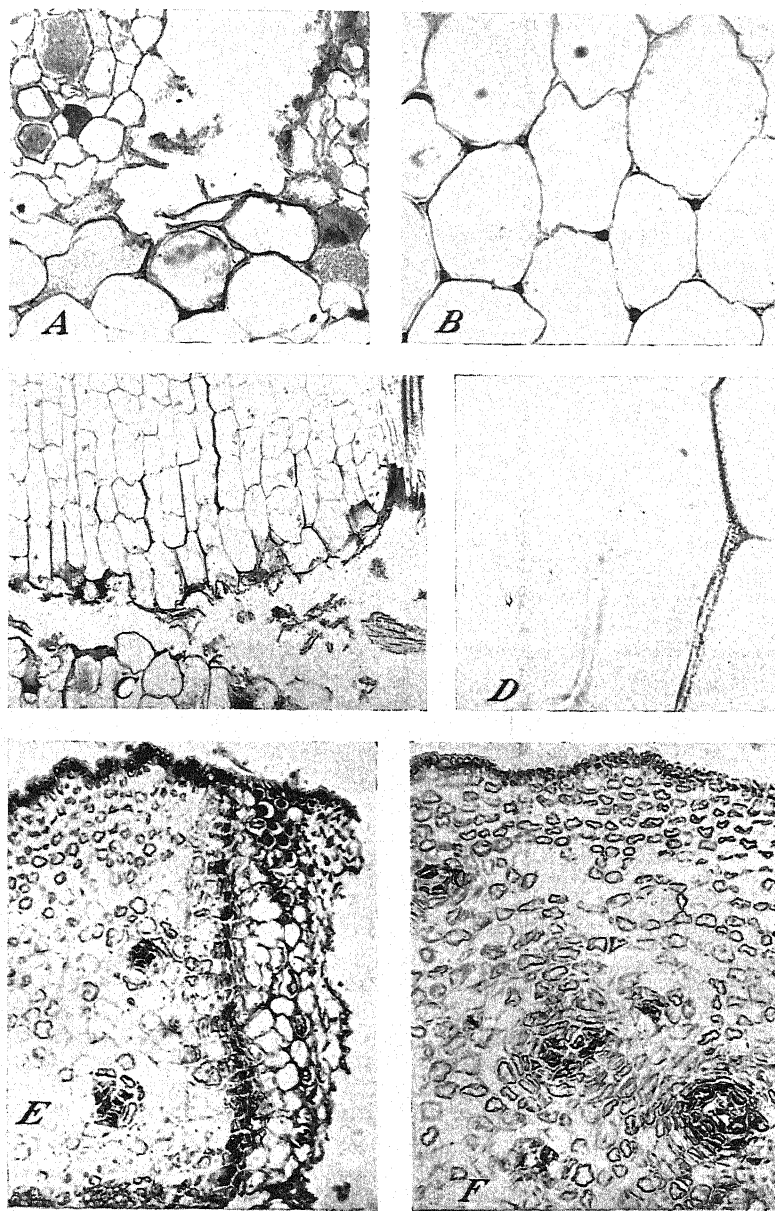
FIGURE 4.—Photomicrographs showing the location of the hairy-root bacteria: *A*, Cross section of a Paris daisy stem 4 days after puncture inoculation, showing the bacteria within injured host cells and in the neighboring vessels. × 270. *B*, Cross section of a Paris daisy stem 8 days after puncture inoculation, showing discolored intercellular spaces which contain the hairy-root bacteria. × 270. *C*, Longitudinal section of a Paris daisy stem 8 days after puncture inoculation, showing discolored intercellular spaces which mark the position of the hairy-root bacteria. × 100. *D*, Longitudinal section of a Paris daisy stem 8 days after puncture inoculation, showing the hairy-root bacteria in the intercellular space. × 550. *E*, Cross section of an apple stem 8 days after puncture inoculation with the hairy-root organism, showing the position of the circular areas of hyperplasia in relation to the host tissues and to the wound. × 200. *F*, Another cross section of an apple stem showing the position of the circular areas of hyperplasia in the host tissues above and below the wounds. × 200.

(fig. 4, *A*). Because no changes in these tissues were observed, the presence of the bacteria in the vessels appeared to be of no importance in bringing about infection.

Several thousand inoculations made on unwounded apple trees failed to produce infection. Symptoms of hairy-root infections induced by inoculation have invariably originated at the point of inoculation. This characteristic of hairy root is in accord with the limited migration of the bacteria from the wound cavity found in the studies on the location of the bacteria.

A study of the location of the bacteria in the older tissue was continued with bacteriological technic.

The bacteria seemed to be most abundant in the soft outer tissues of the enlargement. This was determined from isolation studies in which the writer used the technic described earlier. The interior of the basal enlargements of hairy root consisted chiefly of xylem ele-
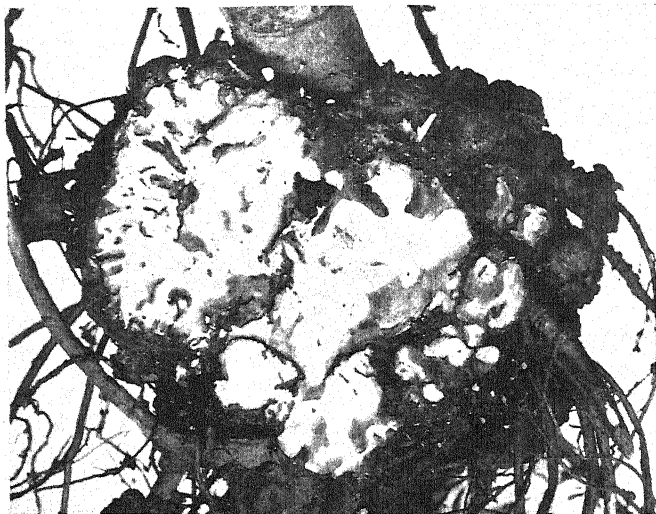
FIGURE 5.—Section of a hairy-root overgrowth after a 15-minute exposure to the air, showing the white, hard interior tissues and the soft outer tissues which have discolored in the air and which usually contain the hairy-root bacteria. Actual size.

ments. For some distance beneath the surface two tissues are paramount in the enlargement—the hard, white, woody interior, and a soft, almost colorless, cortical exterior of varying thickness. Exposure to the air for a few minutes caused these soft outer tissues to become brown as if oxidized. The vascular tissues remained unchanged (fig. 5).

The bacteria were isolated consistently from the surface and subsurface parenchymatous tissues of the enlargements. In order to clarify the position of the bacteria in the enlargements, isolations were made from different tissues. Different kinds of enlargements common to the nursery, approximately 50 percent of which were hairy root and the remainder principally wound overgrowth, were taken at random and isolations made. In a series of 152 isolation trials, 131 series of platings were made from the surface, 152 from the subsurface, and 152 from the deep interior. The surface tissue yielded the largest

percentage of the organism, or 39 percent (table 6). In sampling the surface tissue soil particles were sometimes unavoidably included. The subsurface parenchymatous tissue was removed aseptically and yielded infectious bacteria in 38 percent of the isolation trials. From the deep vascular tissue the bacteria were obtained only from discolored areas where soil particles were imprisoned. It should be noted in the table that nonpathogenic bacteria, *Bacillus radiobacter* Beij. and Van Deld., were uniformly obtained in considerable quantity. The significance of these nonpathogenic bacteria has not been determined. However, the location of the bacteria at the surface of the enlargements is of importance in relation to the soil.

TABLE 6.—*Summary of isolation studies from the different tissues of representative enlargements found in the nursery* [a]

Tissue examined	Isolation trials	Enlargements yielding—		
		Phytomonas rhizogenes	Nonpathogenic bacteria	No bacteria
	Number	*Percent*	*Percent*	*Percent*
Surface parenchymatous	131	39	32	29
Subsurface parenchymatous	152	38	28	34
Deep interior vascular	152	[b] 1	[c] 2	97

[a] No crown galls were included among these specimens.
[b] The deep interior tissue yielded *Phytomonas rhizogenes* only from discolored tissue containing soil particles.
[c] Nonpathogenic bacteria also were obtained only from discolored tissue containing soil particles.

SOIL RELATIONS OF ORGANISM

The bacteria were found to be abundant on the surface of the hairy-root enlargements. The most promising method of studying this problem seemed to be dependent upon the production of infections under approximately aseptic conditions away from common soil contaminants. The production of hairy root under these conditions has been worked out by Riker, Hildebrand, and Ivanoff (*29*), where the technic is given. Examples of the diseased specimens produced by this method are described. Briefly, the technic consisted of fitting glass cylinders around the stems of apple and sealing them so as to exclude all micro-organisms, except the hairy-root bacteria, used in making the inoculations. At intervals of 9, 10, 13, 15, 17, 19, and 21 weeks after inoculation the specimens were taken for study. The surfaces were sterilized by immersion for 15 minutes in a 20-percent solution of a sodium hypochlorite preparation, known as "Bacillikill", which contains 3.5 percent of sodium hypochlorite by weight. The disinfectant was removed by washing through three changes of sterile distilled water. In an effort to recover the organism, bile-agar platings were made of the sterile distilled water in which the specimens were allowed to be immersed for intervals of 10 minutes, 1 hour, 4 hours, 1 day, and 2 days.[5] Hairy-root bacteria were obtained in the washings of all the specimens and for all the washing intervals tested. One control specimen gave off no bacteria; a crown-gall specimen yielded the crown-gall bacteria.

[5] This technic was developed after consultation with W. M. Banfield, Department of Botany, University of Chicago.

A parallel exit study, which was conducted on disease specimens produced in the soil, gave similar results. Four specimens, inoculated, respectively, 2, 4, 6, and 8 weeks previously, were used in this study. These specimens were washed free from soil particles in such a manner as to produce no injuries. They were then sterilized and washed free from the sterilizing solution as in the preceding experiment. The specimens were placed for 1 hour in sterile distilled water before the water was plated. After an incubation period of 1 week the hairy-root bacteria were obtained from the platings of specimens of all four ages. The proof of the identity of the bacteria saved from these isolations was established by positive inoculations on apple.

The bacteria were found rather often in the soil near the hairy-root formations. In 1929, 10 isolation trials were made at each of three 10-day intervals, starting May 28. The technic employed was as follows: For each trial a sample of about 500 g of soil was removed from around a hairy-root formation and taken to the laboratory. After a thorough mixing, a sample of 20 g was weighed into a flask containing 200 g of sterile distilled water. This was shaken and allowed to settle for about 1 hour. One cubic centimeter of the supernatant liquid was then transferred to each of three Petri dishes. Loop dilutions from each of these were made to three other dishes, respectively, containing 1 cc of sterile distilled water. Bile agar was added, and the plates were then allowed to incubate at room temperature for 1 week, after which an examination was made. The percentage yields of the hairy-root organism in the three successive trials of this experiment were 40, 80, and 60, respectively. The bacteria were obtained in 18 of the 30 attempts, or 60 percent.

A similar study, consisting of 10 trials conducted on soil taken from around healthy unions, were all negative. Since the bacteria were obtained relatively easily from random samples from the nursery soil this may seem unusual. However, in securing these samples soil was taken from around trees of a poorly knotting variety where there was less chance of soil contamination.

A 3-day-old growth of bacteria from six large prescription bottles was poured into a cubic foot of soil stored in the field, and the same treatment was given a similar amount of soil kept in a storage cellar. These soils were inoculated in October 1930, and three isolations were subsequently made from each soil at monthly intervals, through April 1931. The soil stored in the cellar yielded the bacteria in 20 out of 21 trials; the field soil yielded the bacteria in 19 out of 21 trials. It is apparent from these experiments that the bacteria may overwinter in soil kept in the field or with nursery stock stored in the cellar.

The longevity of the bacteria in field soil has been found to exceed 1 year both in Wisconsin and in Kansas. At Madison, Wis., steamed soil inoculated with the hairy-root bacteria in the summer of 1928 yielded the bacteria in 2 out of 4 trials in April 1929 and in 1 out of 4 trials in October 1929. Field soil inoculated in the summer of 1929 yielded the bacteria in 1 out of 3 trials in October 1930, and in 0 out of 3 trials in April 1931. In 1930 an attempt was made in Kansas to recover the hairy-root organism from the soil of four fields that had grown or were growing nursery apple trees. Fields 1, 2, 3, and 4 had grown trees during 1927 and 1928, 1928 and 1929, 1929 and 1930, 1930 and 1931, respectively. At five different intervals (June 3, June 17, July 11, July 30, and August 28), 10 soil samples from each

field were collected at random from a depth of about 4 inches and then mixed for each field. Platings were made according to the technic of the previous season, except that four instead of three 1-cc samples were taken for each flask. The results show that the bacteria were present but were not very abundant in three of the fields. Briefly, from a total of 20 trials for each field, the hairy-root bacteria were obtained in 0, 20, 25, and 30 percent, respectively. Only in field 1, from which the trees had been removed 2 years previous, were no bacteria obtained. The presence of the bacteria in field soil from which the trees had been removed a year previous (field 2) and their absence in soil which had grown trees 2 years previous (field 1) indicated the length of time after inoculation that bacteria could be isolated from the soil. Isolations were again attempted in May 1931 from fields 2, 3, and 4 and from the soil of the new graft field, which the season before had grown apple seedlings. Field 2 failed to yield the bacteria, although it had done so the previous season. Fields 3 and 4 and the new graft field yielded the bacteria in 25, 25, and 50 percent of the trials, respectively. The presence of the bacteria in the graft field in May in so short a time after the grafts were planted may perhaps be traced to the growth of seedlings in this field the previous season. These results correspond in general to those secured by Patel (*20*) and Banfield (*3*) for the length of time crown-gall bacteria could survive in unsterilized soil.

DISTRIBUTION AND TRANSMISSION OF ORGANISM

Although nursery stock may be inspected and diseased trees destroyed, it is difficult to find the incipient stages and impossible to find recent infections. Moreover, healthy plants may carry the bacteria on their roots. In 1929 a consignment of apparently healthy Wealthy apple grafts were planted at Madison, Wis. (*23*), in beds of soil that had been steamed. At the close of the growing season some of the trees had overgrowths at their unions. Twenty trees showing these malformations were selected at random. Isolations from 14 yielded typical hairy-root bacteria. The probability that these bacteria entered the trees from the steamed soil is remote, hence they were probably carried either in the unions or on the surface of the roots. It seems fairly obvious that the part played by shipments of nursery stock is important in the widespread dissemination of the disease. This may account for its presence in practically every region where the host plant is grown.

The seedling root was found to be one medium of transmission of the causal organism from one crop to the next. Siegler and Piper (*35*) reported that "the seedlings may in nature carry surface-borne organisms in quantities sufficient to be considered an important source of inoculum." Isolation trials from washings from certain seedling roots have yielded the bacteria. Three-inch pieces from 26 different seedling roots were placed in test tubes partly filled with sterile distilled water. Platings were made from the water 4 hours later. Hairy-root bacteria were obtained from 24, or 92 percent, of the specimens, indicating that the seedling is at least one of the primary sources of inoculum. This and other evidence obtained from these life-history studies points to grafting time as the time when the principal primary infection of the apple tree takes place. The presence in the soil of

primary infections, resulting from the entrance of the bacteria at grafting time, together with the various wound-producing agencies, are the factors which would seem to account for the secondary spread of the disease.

SUMMARY

In studies of infectious hairy root special consideration has been given to the life history of the causal organism in relation to its pathogenesis on nursery apple trees.

The differentiation of hairy root from crown gall and from wound overgrowth has been repeated and confirmed.

The entrance of the bacteria into the host plants was found to be accomplished only through wounds. The bacteria were able to produce infection if placed only on the surface of the injuries.

The type of wound apparently made no difference in the kind of over-growths, but did influence somewhat (1) the length of the incubation period, (2) the percentage of inoculations producing infection, and (3) the size of the reaction. Extremely shallow wounds were found to be poor infection courts.

Wounds on the underground stems of nursery apple trees in the field remained open for infection a relatively short time, averaging about 2 days.

Callus was found ordinarily to be a barrier against the invasion of the hairy-root organism. Shallow wounding of callus only occasionally resulted in infection by the bacteria.

Insects appeared important in producing injuries that led to hairy-root infection. Root-feeding insects have been commonly observed during three growing seasons in the soil about nursery apple trees. During the study of diseased and healthy trees, insects frequently have been seen eating hairy-root and healthy tissue. Infected lateral roots were occasionally observed in positions that could have been reached only by insects. The development of new infections throughout both the first and second growing seasons in Kansas was most easily explained as due to the agency of insects.

Several experiments suggested by the observations on insects indicated a relation of insects to hairy root. The hairy-root bacteria were isolated from white grubs. Isolations from other insects gave negative results. Insect repellents in preliminary trials reduced the amount of hairy-root infection. Insect barriers considerably reduced the amount of hairy root.

The time of the growing season seemed immaterial so far as the entrance of the organism was concerned.

The age of the nursery apple trees in Kansas, where trees are grown only 2 years, did not seem to influence susceptibility to infection by the hairy-root organism.

All sizes of nursery apple trees were found infected in the same field under natural conditions and all sizes became infected when inoculated with the causal organism.

Varietal susceptibility to infection by the hairy-root organism differed widely, ranging from 12 to 100 percent for the 29 varieties studied in 1930, and from 22 to 100 percent for the 37 varieties studied in 1931.

Previous infection of nursery apple trees apparently did not prevent subsequent infection.

The hairy-root organism appeared to begin its relations with the host tissues in the liquid released by the wounds.

The bacteria appeared to be located in the intercellular spaces in both Paris daisy and apple stems.

The presence of the organisms between the cells of the tissues bordering the wounds stimulated a multiplication of the cells in those localities, which resulted in the formation of somewhat circular areas of hyperplasia.

The development of infections without wounds has not been observed on apple trees.

The bacteria were isolated from the surface and subsurface parenchymatous tissues of the enlargements.

The bacteria were found to be abundant on the surface of the hairy-root enlargements.

The bacteria were found often in the soil near the hairy-root formations.

The bacteria overwintered in soil kept either in the field or with nursery stock in the storage cellar. The longevity of the bacteria in field soil which had been steamed or left untreated has been found to exceed 1 year.

The bacteria may be spread long distances by shipments of nursery stock. The seedling root was found to be one medium of transmission of the causal organism from one crop to the next.

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SEASONAL DEVELOPMENT OF HAIRY ROOT, CROWN GALL, AND WOUND OVERGROWTH ON APPLE TREES IN THE NURSERY [1]

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INTRODUCTION

The seasonal development of hairy root, caused by *Phytomonas rhizogenes* Riker et al., crown gall, caused by *Phytomonas tumefaciens* (Smith and Town.) Bergey et al. (synonym, *Bacterium tumefaciens* Smith and Town.), and wound overgrowths on apple trees in the nursery has been studied in relation to certain factors that might influence the initiation, development, and control of these diseases. This work was undertaken as a part of the program on the complex graft-knot problem. After the differentiation of these various diseases from one another, a successful effort was made by the senior writer and his associates (*8, 10*)[3] to induce them at will under controlled conditions. A study was then made by Hildebrand (*4*) of the life history of the hairy-root organism in relation to pathogenesis, with special emphasis on the mechanism of infection. In connection with these lines of work, it appeared desirable to know at what times these overgrowths on nursery apple trees were initiated, under what conditions they developed, and the relative importance of the infections periods.

This study was made in eastern Kansas near the center of the region where piece-root apple grafts are planted in large numbers, where apple seedlings are grown, and where hairy root has occurred with a frequency satisfactory for detailed study. Control measures which had proved adequate in other places often either failed or were only moderately successful in this locality. This fact emphasized the value of the location for studying one of the most difficult phases of control; consequently the analyses presented are of a severe rather than an average situation. The observations and experiments carried out in considerable detail in eastern Kansas were correlated with findings in other nurseries. At least once every fall the results were compared with those secured in Iowa, Minnesota, Missouri, Nebraska, Kansas, Oklahoma, and Wisconsin.

A preliminary statement of some of the earlier phases of this work has already appeared (*11*).

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EXPERIMENTAL PROCEDURE

The procedure followed in these studies is an adaptation of that described by Keitt and Jones (5). The methods used were selected in consultation with Dr. Keitt.

ENVIRONMENT

The histories of the fields employed varied somewhat. The field in which experimental grafts were planted in 1929 had carried crops of potatoes in 1926, 1927, and 1928. The field in which experimental grafts were planted in 1930 had previously grown three successive crops of corn. The field which was used for experimental grafts in 1931 had grown corn in 1928 and 1929 and apple seedlings in 1930.

The soil reaction was tested from time to time in the three fields in which experimental grafts were planted. The reaction was approximately pH 5.0 in all the fields. The different tests showed comparatively little variation.

Air temperatures were recorded by a thermograph housed 4 feet above the ground in an instrument shelter located among the first-year experimental trees.

The relative humidity of the air was recorded by a hygrograph corrected by the use of a sling psychrometer (U.S. Weather Bureau pattern) and Marvin's (6) psychrometric tables.

Soil temperature at the level of the graft union, between 4 and 5 inches beneath the soil surface, was recorded with a soil thermograph. All the instruments were checked daily.

Soil-moisture samples were taken daily from about 4 inches below the surface. The water in the soil was measured in terms of the moisture-holding capacity. With a technic like that used by Riker (7), the moisture-holding capacity of the soil in each of the three experimental fields was found to be approximately 38 percent of the oven-dry weight. The amount of water in the daily samples was measured by Bouyoucos' (3) rapid method and calculated in terms of the moisture-holding capacity.

The rainfall for each 24-hour period was measured with a rain gage having a 3-inch cup.

HOST DEVELOPMENT

The plant used for all of these studies was the Yellow Transparent variety of apple. Since the diseases studied occur most frequently at the union, the experimental trees were all grown from piece-root grafts made with Kansas seedlings.

The factors of host development considered were the height of the tree and the diameter near the ground level. Measurements were taken at 2-week intervals on 300 first-season and 300 second-season trees. The same trees were used for successive measurements. The height in inches was measured from the original scion where growth began to the top of the main stem. After the early stages of development had passed, the lower point corresponded rather closely with the soil level and was chosen to avoid variations in measurements due to changes in the soil level incident to cultivation. The diameters of the trees in sixteenths of an inch were measured with a nursery-man's caliper about 1 inch above the point from which the new growth left the original scion. This location on the stem was chosen because it was easy to find, was usually just above the ground level, and was generally the place where the stem had the greatest diameter.

STIMULATION OF OVERGROWTHS

Nonparasitic overgrowths were induced in two ways. In some cases aluminum-alloy wire was wrapped twice about the scion a short distance above the union. In other cases a cut about half-way through the scion was made with an upward pull of the knife so that a wedgelike projection of tissue extended down. Direct healing was prevented by inserting a strip of adhesive tape in the cut. This cut produced a result similar to that often found in a poorly fitted graft.

The parasitic overgrowths were induced by inoculations with pure cultures of the causal bacteria. Crown gall was induced with culture A–1 of *Phytomonas tumefaciens*, which was grown from an individual cell. Hairy root was induced with culture C–1 of *P. rhizogenes*. This culture was also grown from a single cell. An account of the purification of these cultures and of their bacteriological characters has already appeared (*14*).

Inoculations at grafting time were made by smearing the cut surfaces of the scions and roots with a suspension of bacteria before these parts of the graft were fitted together and the union was wrapped.

The experimental blocks of trees consisted of a series of parallel rows, each row of which was used for the study of a certain factor, as explained later. Thus on a given day 10 trees in the first row were inoculated with the crown-gall organism, 10 trees in the second row were inoculated with the hairy-root organism, 10 trees in the third row were inoculated with a mixture of the hairy-root and crown-gall organisms, 10 trees in the fourth row received control wounds, 10 trees in the fifth row were girdled with wire, and 10 trees in the sixth row were cut and taped to stimulate wound overgrowth. On successive weeks during the season similar treatments for the production of the overgrowths were made on adjacent trees under comparable conditions.

The method of inoculation during the growing season was as follows: A trench to the depth of the union was made in the soil about 3 inches away from the trees on one side of the nursery row. The trench was opened this distance away to prevent injury to the main stems of the trees while digging. The soil was removed from around the individual trees with a dibble and by hand. Any particles that remained on the main stem were wiped away. Inoculum from a culture of the bacteria was applied with a cotton swab to the stem surface, usually in five places spaced about 1 inch apart. With a scalpel held at an angle, two thrusts were made through each of the drops of culture deep into the stem. Control wounds were made in a similar manner except that no bacteria were employed. During the 1930 and 1931 seasons strips of adhesive tape were applied over both the inoculated and the control wounds to reduce chance contamination from the soil and to keep out insects. Promptly after inoculation the soil was thrown back into the trench so as to cover the topmost wound by about 2 inches.

DEVELOPMENT OF ARTIFICIALLY INDUCED OVERGROWTHS

After the inoculations attention was given to the incubation periods necessary for the development of distinct symptoms.

The incubation period for crown gall was determined by making inoculations at weekly intervals throughout the season with a single-

cell culture, A–1, of the crown-gall organism. In 1929 the first inocula-
tions were made, as described earlier, on May 15 and the last on
September 11. The corresponding first and last inoculation dates
for 1930 were May 12 and September 8, respectively, and for 1931,
May 11 and September 14. Because of the similarity in appearance
of callus, crown gall, and hairy root, especially in the early stages, the
inoculations were not considered positive until the crown galls had a
radial extension of 4 mm. This is in accord with the criteria used by
Riker, Hildebrand, and Ivanoff (*10*). The incubation time given in
the charts (figs. 1, 2, and 3) for any one period is the average time
required for the positive reactions from 50 inoculations. Minimum
and maximum times are recorded in figure 4. Approximately 55
percent of the inoculations gave positive reactions.

The development of crown gall after inoculation was noted at
weekly intervals for the first 6 weeks, during the early disease stages,
and at biweekly intervals thereafter. The number, character, and
radial extension of the overgrowths were recorded at successive
intervals during the season.

The incubation period of hairy root was determined after making
inoculations with a culture (C–1) grown from a single cell of the hairy-
root organism. The methods and intervals of inoculation were the
same as for crown gall. The incubation period for hairy root was
recorded in two ways: (1) The time required for a radial extension of
3 mm and for the appearance of root primordia is indicated on the
charts by a small circle in the line indicating the incubation period,
and (2) the time required for the development of roots 1 cm long is
shown at the end of the incubation period. The incubation time
given for any one period is the average time required for the positive
reactions from 50 inoculations. The minimum and maximum times
are recorded in figure 4. Approximately 70 percent of the inocula-
tions gave positive results.

Similar studies were made of the incubation periods and develop-
ment of the reactions when mixtures of crown-gall and hairy-root
bacteria were used for the inoculum.

The host reactions to uninoculated control wounds made in parallel
series were examined as controls on the hairy-root and crown-gall
series. The procedure was the same except that no bacteria were
employed.

The host reactions to wire girdles and to knife cuts with tape inserts
were followed in parallel series and at similar time intervals.

The development of disease from inoculations with the hairy-root
bacteria at grafting time of 300 string-wrapped and 300 tape-wrapped
grafts was observed at monthly intervals during 1930 and 1931.

NATURAL OCCURRENCE OF OVERGROWTHS

The natural occurrence of hairy root was determined in 1929
through biweekly examinations of the unions of 300 trees grown from
string-wrapped grafts. In 1930 and 1931 monthly examinations were
made of the unions on 1,200 trees, including 300 first-year and 300
second-year trees grown from string-wrapped grafts, and 300 first-
year and 300 second-year trees grown from tape-wrapped grafts.
Observations were made at stated intervals by removing the soil
from about the union in the manner previously explained. If a tree
was accidentally injured in the process, this fact was noted and data

were taken accordingly. At the end of the experiments comparisons were made between the percentages of overgrowths on trees examined at various intervals and on those left undisturbed. The variations ranged from 0 to 10 percent. Consequently, it appears that injuries incident to the periodic examinations introduced little if any error into the results secured.

<center>INSECT RELATIONS</center>

Insect surveys were made of the soil in which the experimental trees were growing. In 1929 the work was begun intensively on May 5 and carried on until July 12, when it was discontinued. Subsequently only insects that appeared in connection with the other work were collected and preserved. However, the survey was carried through the season in 1930 and 1931. At biweekly intervals three areas of soil 9 inches square and 6 inches deep were taken at random from around three first-year trees. These samples were mixed and one third of the mixture was taken to the laboratory for examination. All insects observed while the larger sample was being divided were also taken. The soil was minutely gone over by spreading it on light brown paper. The insects present were collected and placed in vials containing 70-percent alcohol, for later identification.[4] The observations in 1930 were begun May 29 and finished September 18. The first examination in 1931 was on May 11 and the last on September 29.

<center>EXPERIMENTAL RESULTS</center>

The more significant aspects of the results obtained are considered under the following heads: (1) Seasonal variations, (2) incubation periods, (3) appearance of overgrowths at different stages of development, and (4) date of natural infection. The records are given in figures 1 to 5, inclusive.

<center>SEASONAL VARIATIONS</center>

Seasonal variations were large during the 3 years covered by these studies. These variations provided opportunities for observing the effect of seasonal changes both at different times in the same season and at corresponding dates in different seasons (figs. 1, 2, and 3). Since the details are recorded in the figures, only the grosser aspects are discussed here.

The air temperatures during 1929 at Topeka, Kans., were generally below those of the average year in that locality. On the other hand, the air temperatures during the season of 1930 were generally above normal, favorable growing temperatures extending into late October. Although the season of 1931 was considerably warmer in June than the previous two seasons, it was about normal in July, below normal in August, and considerably above normal in September.

The average air-humidity readings for the season of 1929 were much higher than those of the two subsequent seasons. During 1930 the average air humidity was the lowest of the three seasons. But for the month of June the 1931 season had an average air humidity falling between those of the preceding two seasons.

The soil temperatures at the level of the graft unions quite naturally followed the trends of the air temperatures. During the day the soil temperatures lagged behind the air temperatures, but at night the

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[4] The identifications were made by C. L. Fluke, Jr., and E. M. Searls, of the University of Wisconsin.

opposite relation occurred. As was expected, the extremes of soil temperature were much closer together than those of air temperature.

Soil-moisture data were closely correlated with those of soil temperature and rainfall. During 1929, because of the relatively low soil temperature and high average rainfall, the soil-moisture values were generally the highest of the three seasons. In 1930, because of relatively high soil temperature and low average rainfall, the average soil humidity was the lowest. High soil temperature in June 1931 was accompanied by low soil moisture, largely, perhaps, because of the extremely low rainfall during that month. In September, because of relatively high soil temperature and relatively heavy rainfall, the soil moisture ran a middle course.

Rainfall was rather variable during the three seasons. It was above normal in 1929 and below normal in 1930. In 1931 it was below normal in May and June and approximately normal the remainder of the season.

The host development of nursery apple trees for the 1929 season, according to the local nurserymen, was the largest of any season over a period of 40 years, despite the fact that the trees went into dormancy earlier than in the two following seasons. The large growth was accomplished under conditions of higher soil moisture and somewhat lower temperature than usually obtained. There was no slack period due to soil moisture or temperature conditions. The growth of the trees in 1930 suffered because of the drought and abnormally high temperatures in July and August. However, considerable growth was made during September and October, which partially offset the less favorable period. In 1931 the less favorable period for host growth was in June, when abnormally high temperatures and low soil moistures prevailed. Because of a well-distributed rainfall throughout the remainder of the season the trees overcame much of the handicap of July and August and approached the large growth of the 1929 season. The high temperatures and normal moisture conditions of late fall enabled the first-year trees to make more than average growth, and the second-year trees to make the largest growth of the three seasons. The height and caliper measurements were closely correlated during the three seasons, with one exception. The early fall of 1929 caused the trees to stop growth in height in late September, but the growth in diameter continued into October. Records of the growth of the trees are given in figures 1, 2, and 3.

Insect surveys showed that a number of different kinds of root-chewing insects were present during the growing seasons of 1929, 1930, and 1931. Since the larvae only were found in most instances, it was not possible always to determine the species or even the genus. According to information received from C. L. Fluke, Jr., and E. M. Searls, of the University of Wisconsin, the insects considered most important from the standpoint of opening infection courts were the white grubs (Phyllophaga), wireworms (Elateridae), and fungus gnats (Mycetophilidae). White grubs and wireworms were continually present, but were more abundant in June, early July, and September than in May and August. Fungus gnats were more commonly associated with crown gall and were less generally present than the white grubs or wireworms. From these studies it appears that various insects capable of opening infection courts for crown-gall or hairy-root bacteria were present in varying abundance throughout

the growing seasons. The relation of insects to crown-gall infection on raspberries has been discussed by Banfield (*1, 2*). A similar relation to hairy-root infection on apple trees has been investigated by Hildebrand (*4*).

<div align="center">INCUBATION PERIODS</div>

The time required for the development of excess callus and wound overgrowth following wire girdling or cuts was 2 weeks or more, depending upon the rapidity of the growth of the plant.

<div align="center">CROWN-GALL ORGANISM</div>

The incubation periods of the crown-gall organism were on an average much longer in the earlier and later parts of the season than in the middle. An inoculation was considered positive only when the gall had a radial extension of 4 mm after 3 weeks (fig. 8, *F*). The minimum and maximum periods are given in figure 4. Doubtless larger numbers would have produced more regular curves. Charts giving the average incubation periods (figs. 1, 2, and 3) show what appears to be a rather close correlation between short incubation periods and warm weather. Only slight differences were noticed in the length of the incubation periods for corresponding weeks in the different growing seasons.

These incubation periods are concerned only with vigorously growing trees. Inoculations on trees which were making little or no growth did not induce disease until the trees began to develop. Inoculations on such trees were often negative, but reactions have sometimes appeared after normal incubation from the time growth began. Thus after inoculations made late in the season overgrowths might not appear until the following spring. The incubation periods on trees growing under favorable conditions had a definite maximum period after which no infection appeared and which seemed to be correlated with the rapidity in growth of the tree and with temperature.

<div align="center">HAIRY-ROOT ORGANISM</div>

The incubation periods for the hairy-root organism were followed in the same way as for the crown-gall organism. The averages are shown in figures 1, 2, and 3, and the minimum and maximum periods in figure 4. The average length of time necessary for development of roots 1 cm long ranged from approximately 3 weeks in the warm period of the growing season to 9 or more weeks in the cool periods at the beginning and end of the season. Two stages in the incubation period of hairy root were observed. The first stage, indicated by a circle on the charts (figs. 1 to 3), was reached when the average hairy-root enlargement had a lateral extension of approximately 3 mm and when root primordia were beginning to show (fig. 6, *A*, after 3 weeks). The second stage in the incubation period was reached when the hairy roots had a lateral extension of 1 cm (fig. 6, *A*, after 5 weeks). As in the case of crown gall, only slight differences were noticed in the length of the corresponding hairy-root incubation periods in the different seasons.

Experiments were conducted to determine when disease would develop from inoculations made at the time of grafting. These trials were made in 1930 and 1931 on both string-wrapped and tape-
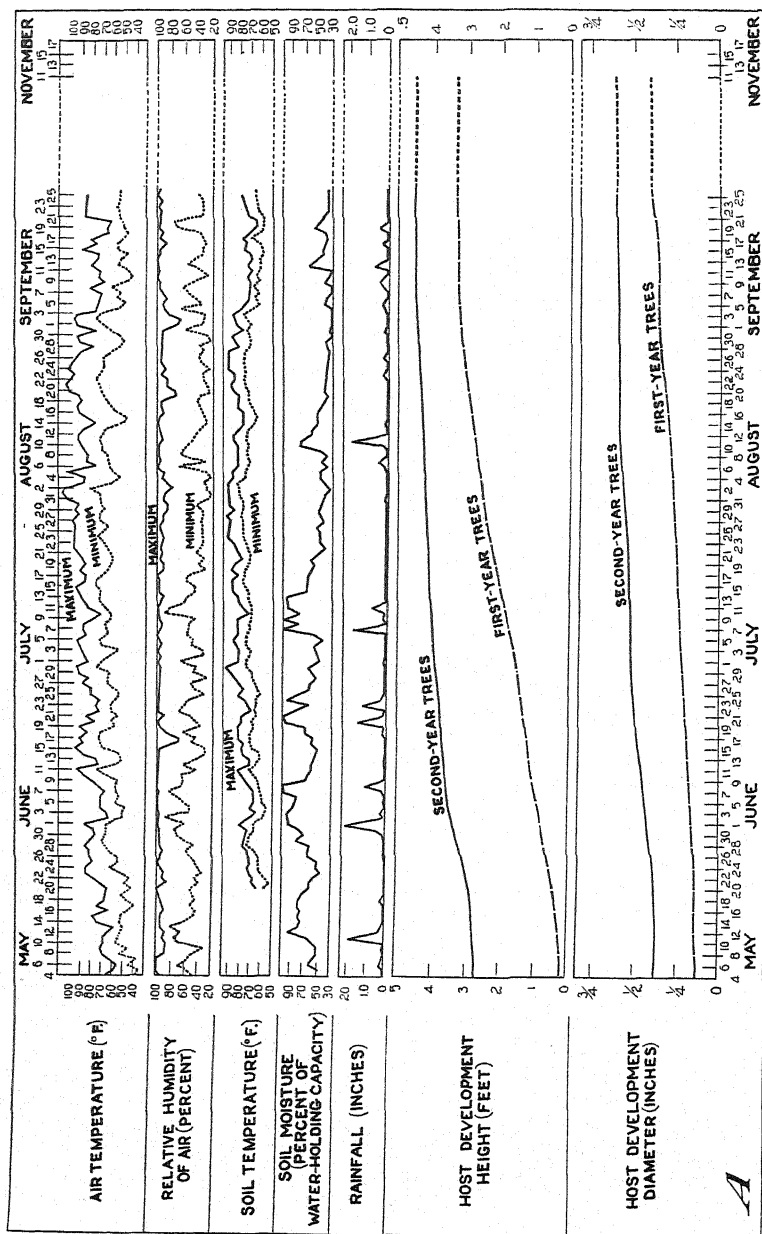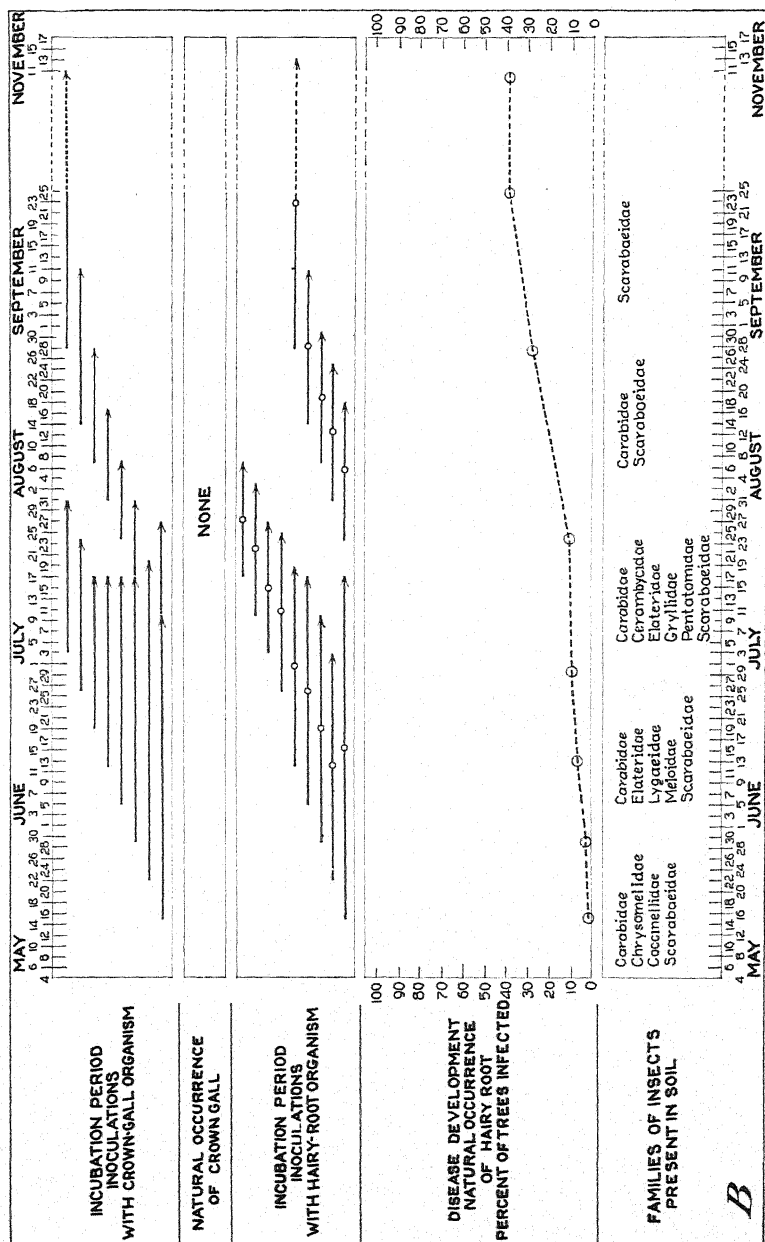
FIGURE 1.—Summary of data relating to seasonal development in 1929 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.
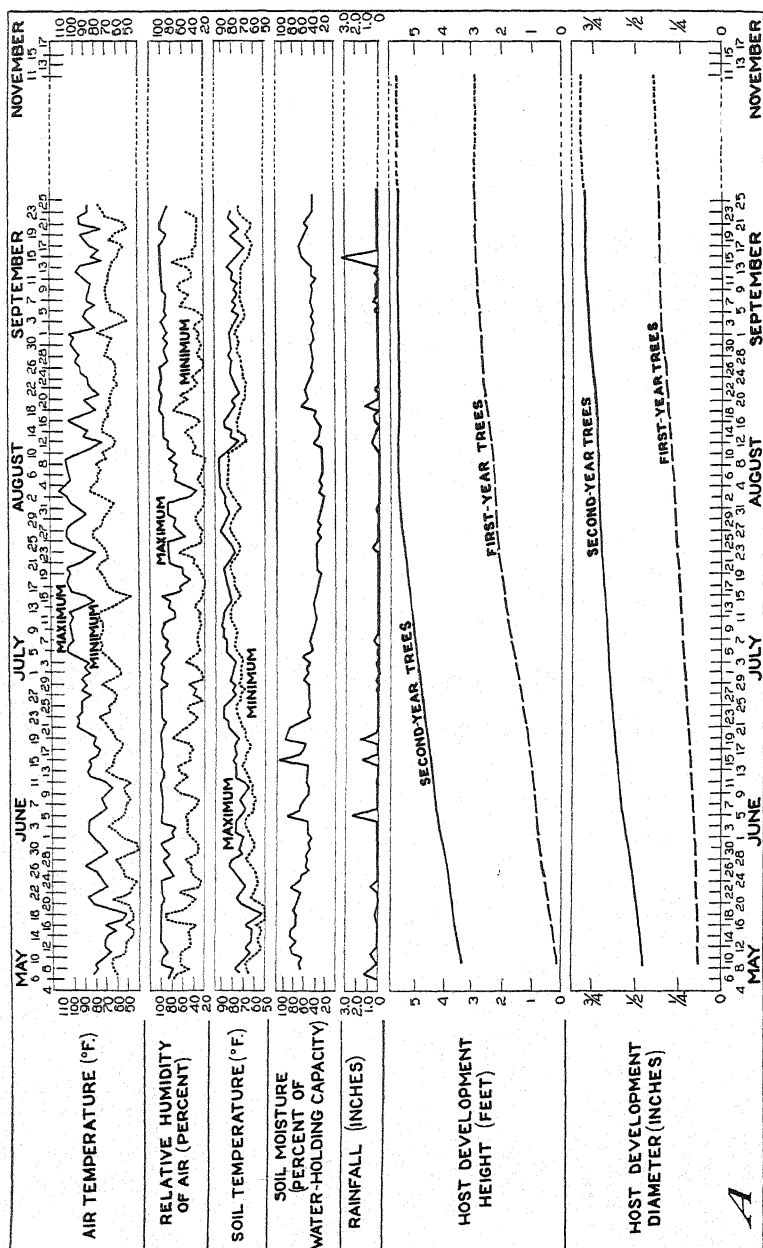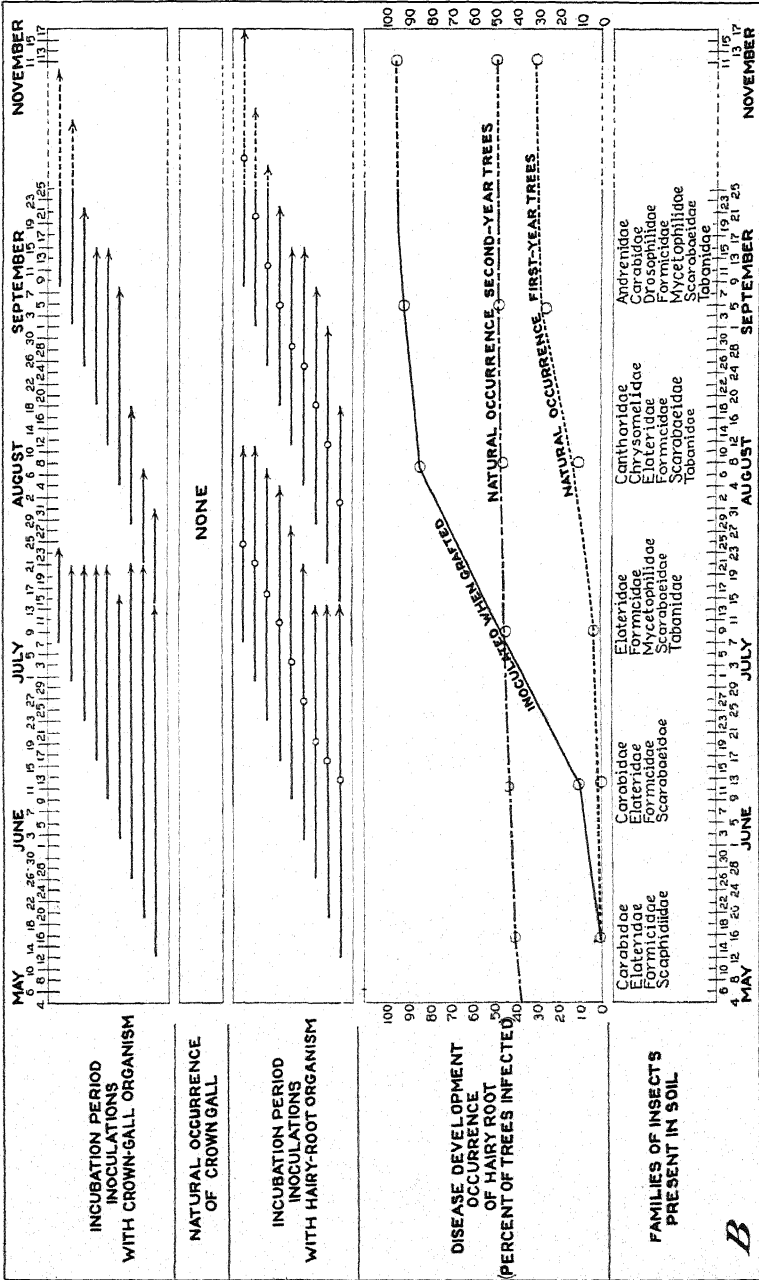
FIGURE 2.—Summary of data relating to seasonal development in 1030 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.
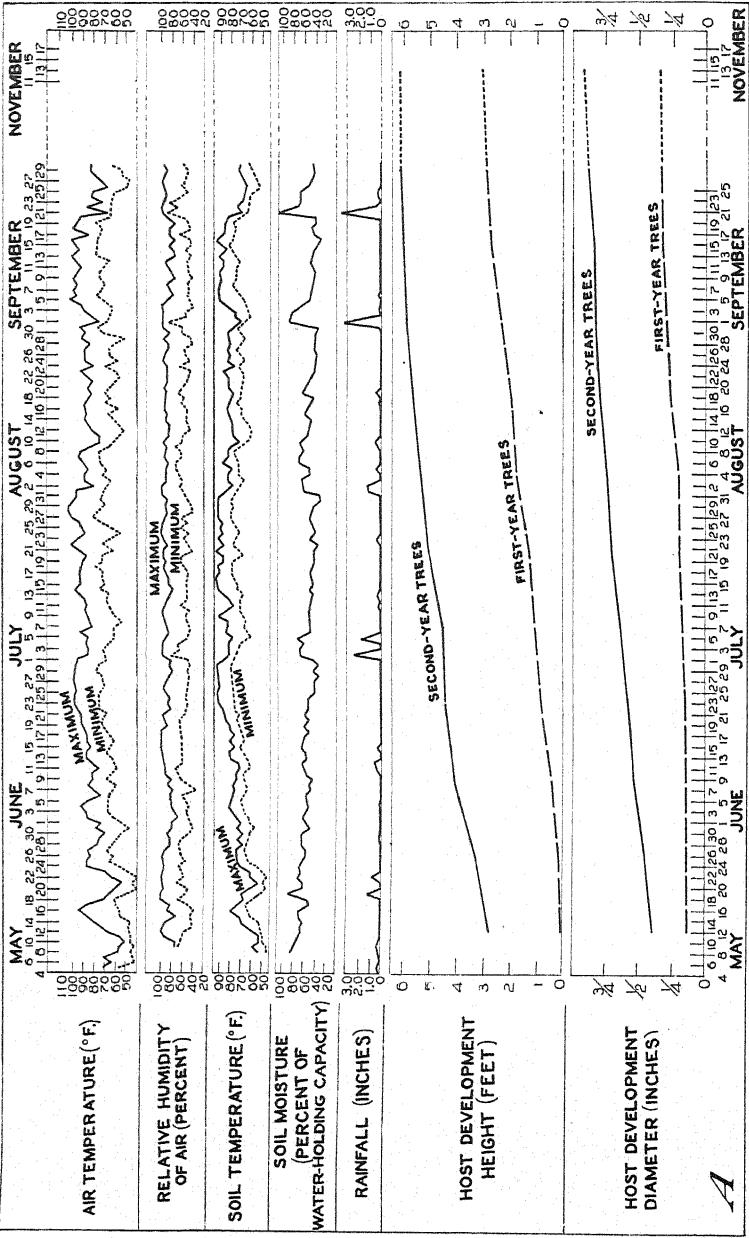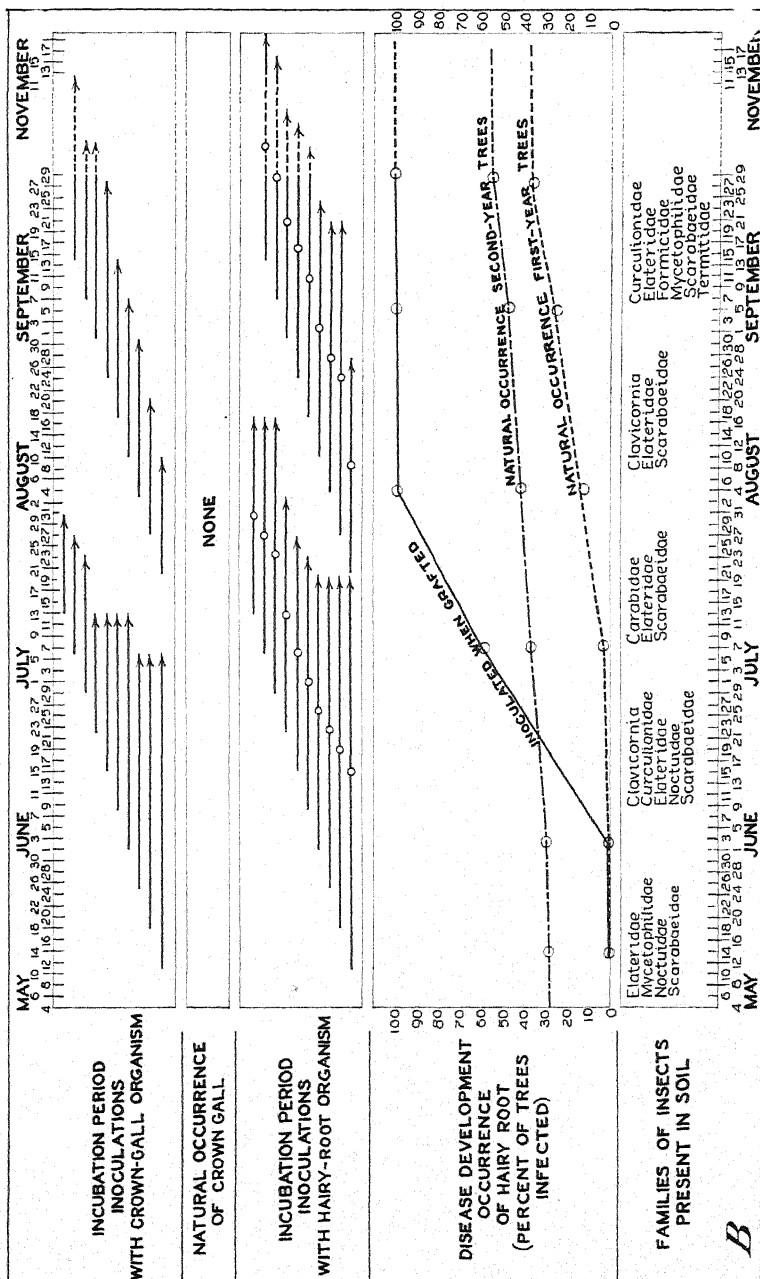
FIGURE 3.—Summary of data relating to seasonal development in 1931 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.

wrapped grafts. The results are recorded in figures 2, 3, and 5. The average time at which hairy-root symptoms (i.e. roots at least 1 cm long) developed in the string-wrapped grafts appeared to be the early part of July in both 1930 and 1931. The minimum was early in
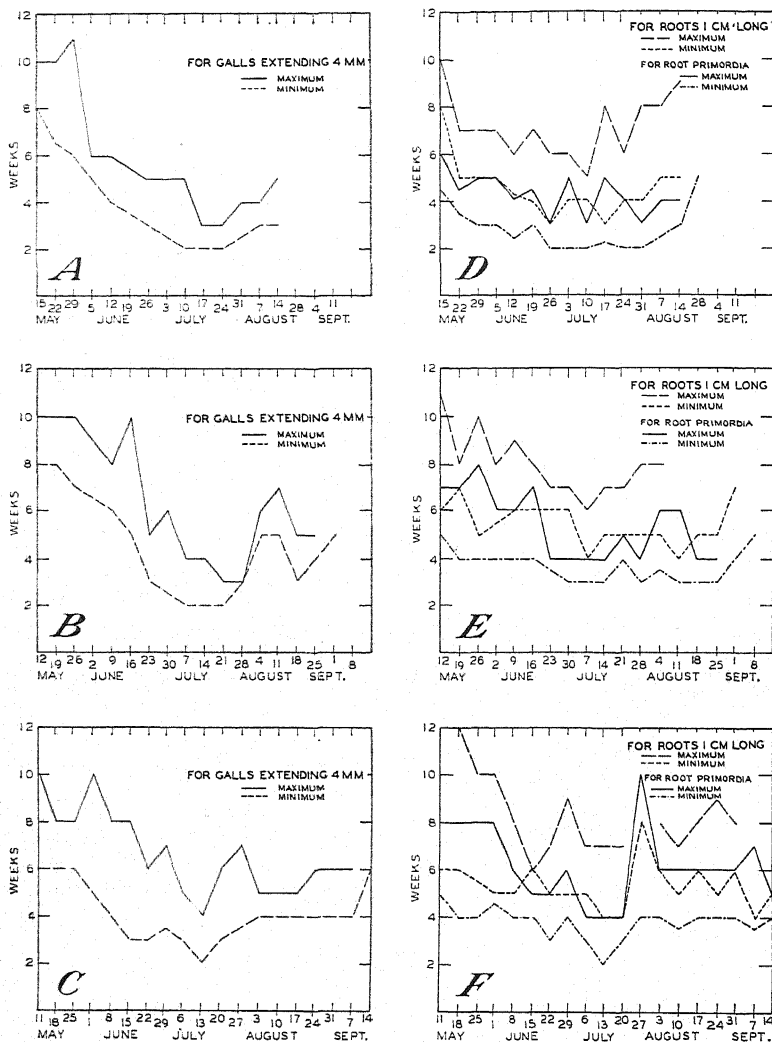
FIGURE 4.—Progressive variations in the length of incubation periods following inoculations made, on the dates indicated, with crown-gall and hairy-root bacteria during the active growing seasons of 1929, 1930, and 1931. The characters of the overgrowths indicating a positive reaction are explained in the text: *A*, Crown gall, 1929; *B*, crown gall, 1930; *C*, crown gall, 1931; *D*, hairy root, 1929; *E*, hairy root, 1930; *F*, hairy root, 1931.

June. The maximum was difficult to determine because of the occurrence of natural infection, which complicated the results. Most of the grafts showed infection by early August. As judged from other inoculations, the maximum probably did not greatly exceed this.
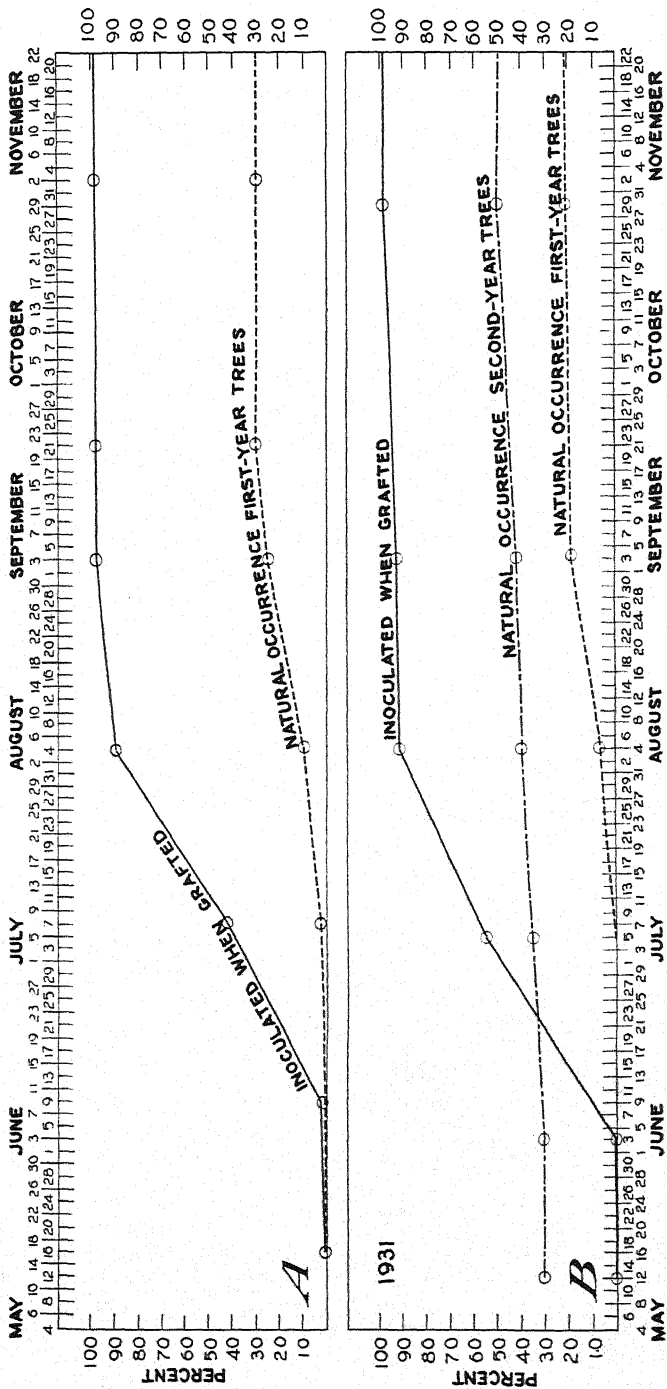
FIGURE 5.—Summary of data relating to hairy-root development in 1930 (*A*) and 1931 (*B*) on nursery apple trees grown from tape-wrapped piece-root grafts.

The occurrence of hairy-root symptoms, following inoculations at the time the tape-wrapped grafts were made, was studied in parallel series. In 1930 only 2 percent of the inoculated grafts had shown symptoms by the middle of June. Hairy root was noted on 41 percent of the grafts on July 9, on 90 percent early in August, and on 97 percent early in September. In 1931 none of the grafts showed symptoms when examined early in June. Early in July, 54 percent showed hairy root; early in August there was 91 percent; and by the end of the season there was 99 percent. Like the string-wrapped grafts, the tape-wrapped grafts that were inoculated when made showed hairy-root symptoms early, during the season of long incubation periods, whereas symptoms of hairy root resulting from natural infection appeared later, during the season of relatively short incubation periods. The tape wrapping had little if any influence in preventing infection when inoculations were made at grafting time. Little if any correlation was noted between the time of the occurrence of hairy root following inoculation at grafting and the time of the natural occurrence of the disease.

The strength of the tape wrappers was found to diminish gradually, until the cloth broke under the slightest strain. Where the wrapping was overlapped the cloth lasted much longer. Frequently the tape wrappings had lost their strength by June, so that the hairy-root developments from inoculations easily came through. Sooner or later the wrappings were cracked open by the growth of the trees. This process began in June and progressed with varying rapidity, depending upon such factors as the moisture and temperature of the soil, the amount of overlapping of the wrapper, and the increase in diameter of the tree. It is clear that while the wrapper remained intact, the union was protected against root-chewing insects.

APPEARANCE OF OVERGROWTHS AT DIFFERENT STAGES OF DEVELOPMENT

Hairy root, crown gall, mixtures of these two, and callus or wound overgrowth were studied as they developed after inoculations or special treatments made at different times. The results of these extended studies conform in general with those obtained in limited trials by Riker et al. (*9*). Since a complete new series of tests was started each week during three active growing seasons, the volume of material available was large. Consequently the development of only one representative series for each type of overgrowth is considered here.

The symptoms of the different enlargements in the various stages of development are illustrated in figures 6 to 8. The descriptions given are of typical symptoms that followed the different treatments administered during the latter part of May.

HAIRY ROOT

Hairy-root development is described first for one of the series made during 1930. The cultures and methods of inoculation employed have already been given. The development of the various symptoms was more rapid on the Yellow Transparent trees in Kansas than on Wealthy trees in Wisconsin (*9*).

After 1 week the scalpel cuts in which the inoculations were made showed practically no change from the control scalpel cuts. A slight
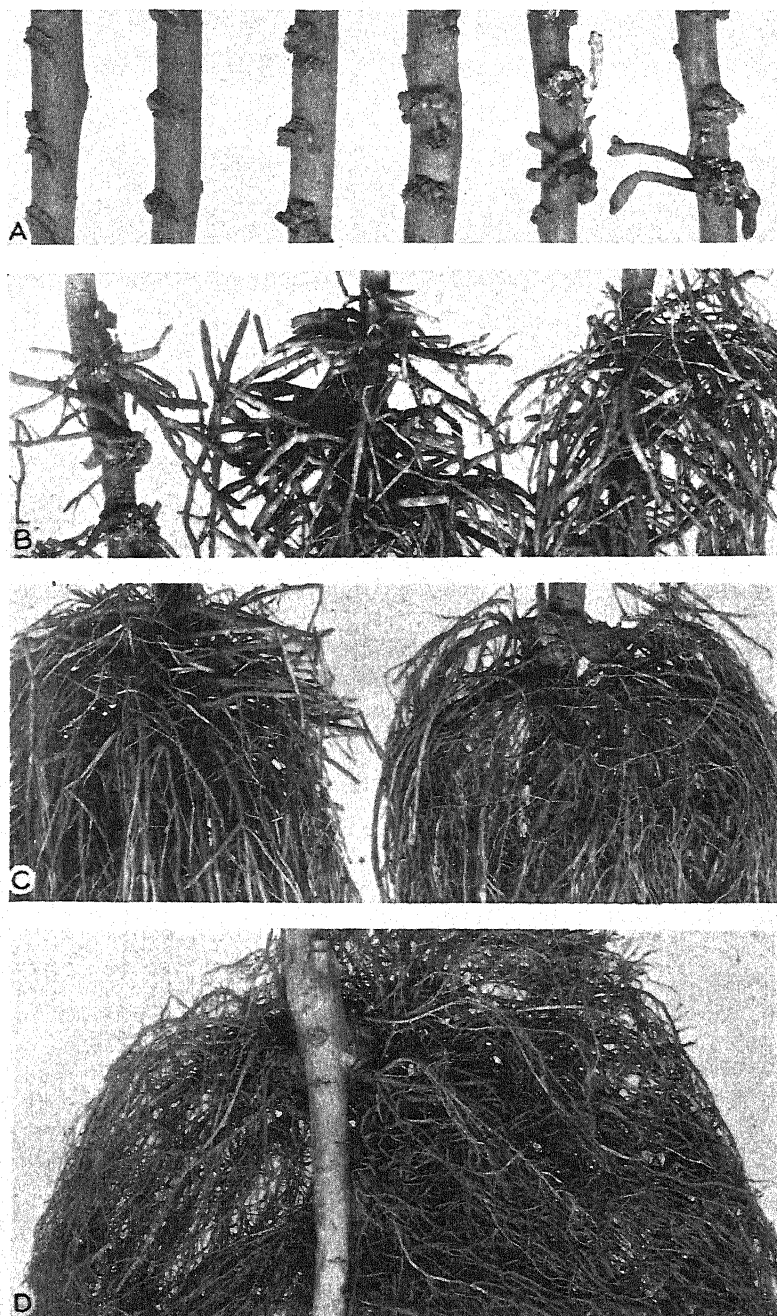
FIGURE 6.—Hairy-root symptoms after wound inoculations, as they appeared during the first year on scion wood below ground: *A*, After 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right; *B*, after 8, 10, and 12 weeks; *C*, after 16 and 18 weeks; *D*, after 24 weeks.

formation of wound tissue was apparent, especially about the exposed portions of cambium.

After 2 weeks the frosty calluslike tissue had so increased in size that it practically covered the injury. Small hemispherical frosty knobs of new tissue appeared sometimes to extend from the callus. No difference was noticed between the inoculated and control wounds. The lateral extension of the tissue was approximately 2mm.

After 3 weeks the hairy-root tissue had grown until it had an average lateral extension of about 3mm. This was somewhat greater than

FIGURE 7.—Hairy-root symptoms after wound inoculations, as they appeared during the second year on scion wood below ground: *A*, In May. Many of the smaller roots were killed by frost.; *B*, In August. Certain of the roots appear much larger than others. *C* and *D*, In October. Wound-overgrowth tissue appears in conjunction with that of hairy root.

the callus at the control wounds. At this time small, more or less round protuberances of tissue, root primordia, approximately 2 mm in diameter, made their appearance from the surface of the basal tissue. Often, but not always, these appeared to be further developments of the knobs mentioned earlier. This stage is indicated by circles in the arrows of the charts designating incubation periods (figs. 1, 2, and 3).
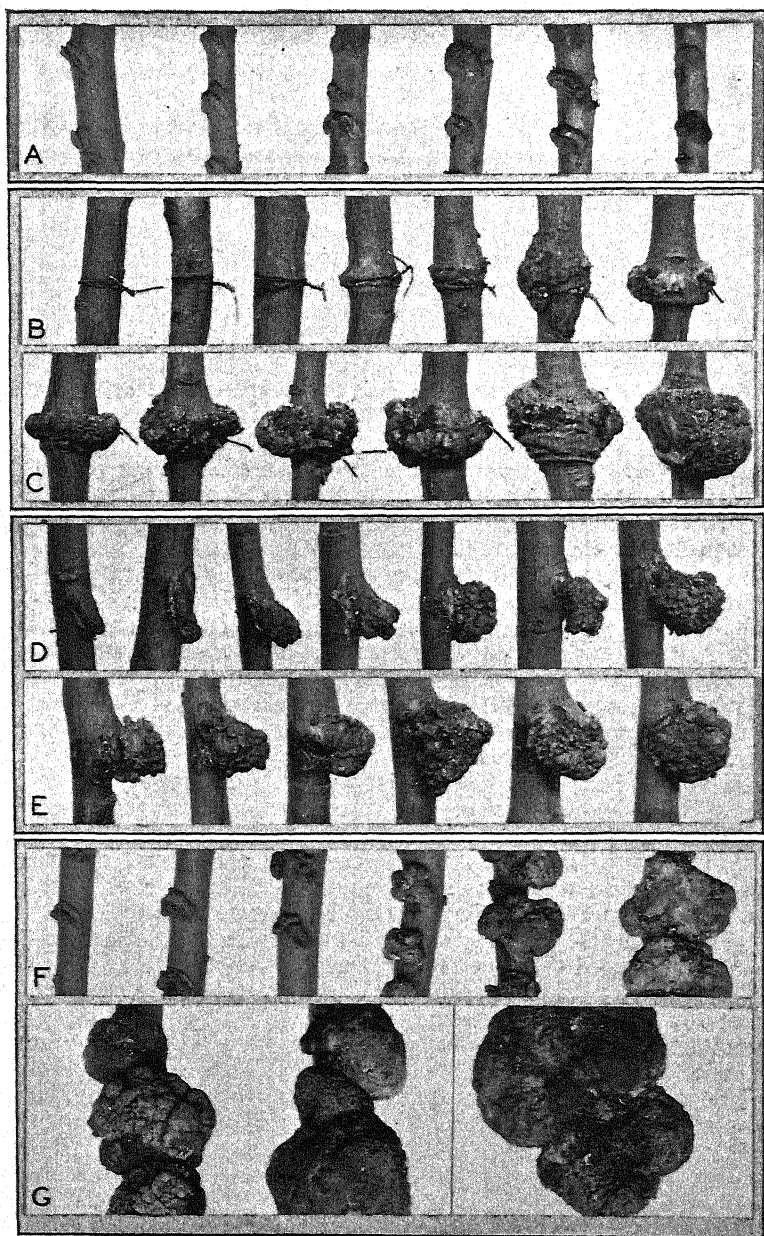
FIGURE 8.—Several types of overgrowths after various treatments, as they appeared during the first year on scion wood below ground: *A*, Wounds like those used for inoculation, except that no bacteria were employed, after 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right; *B*, induced by wire girdles, after 1, 2, 3, 4, 5, 6, and 8 weeks, respectively, from left to right, and (*C*) after 10, 12, 14, 16, 18, and 20 weeks; *D*, induced by knife cuts, into which were inserted bits of adhesive tape, after 1, 2, 3, 4, 5, 6, and 8 weeks, respectively, from left to right, and (*E*) after 10, 12, 14, 16, 18, and 20 weeks; *F*, crown galls that followed wound inoculations as they appeared after 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right, and (*G*) after 8, 10, and 12 weeks.

After 4 weeks both the basal tissue and the protuberances had enlarged until the average lateral extension was approximately 4 mm. Some of the protuberances had taken on more definitely the appearance of root primordia. In exceptional cases small fleshy roots were starting.

After 5 weeks the basal enlargements had increased a little and root primordia were quite common. Small fleshy roots were also more frequent. The root primordia and roots gave the surface a rough and irregular appearance, in contrast with the relatively smooth surface of crown galls. Some of the small roots were over 1 cm long, indicating the final stage of the incubation period.

After 6 weeks the number of fleshy roots was decidedly greater, and the older ones had reached a length of 2 cm. After this time the basal enlargements increased in size very slowly and some became more or less completely covered by the root developments.

After 8 weeks the fleshy roots had increased considerably both in number and in length. Many had begun to branch, and a few small fibrous roots were found.

After longer periods during the first season following inoculation there was a progressive increase in the number and length of the hairy roots. Large masses of both fleshy and fibrous roots were very common at the end of the growing season. The basal enlargements were ordinarily rather inconspicuous the first year and were well covered with root developments.

The amount of moisture in the soil appeared to be an important factor in the development of the roots. Inoculations made aboveground or in the upper portions of the soil, where it frequently became dried out, showed little or no root development. Evidence was frequently found that roots had started but had been killed by subsequent drying of the surface soil.

The vigor of the trees likewise appeared to influence the development of the hairy-root symptoms. Grafts which had not become well established and had not produced new growth at least a foot high made little or no response to hairy-root inoculations. The relation between the size of tree and the incidence of hairy root at digging time has been more fully discussed by Hildebrand (4).

The period in the growing season when the inoculations were made likewise influenced the rapidity with which the symptoms developed. The incubation periods for different stages of the disease were shorter during the summer than in the spring or fall. In some cases when inoculations were made in September, no symptoms were observed until the following year. Although the time at which they appeared varied considerably, the sequence of symptoms remained the same.

During later development after inoculation the hairy-root overgrowths showed a somewhat different character (fig. 7). The roots that were actively growing when cold weather set in and the ground became frozen were killed. However, those that had reached sufficient maturity were able to withstand the winter and to continue their activity the second year. By June of the second year, certain of the larger roots had grown considerably, both in length and diameter. Many small lateral roots appeared that were more likely to be fibrous than fleshy. However, fleshy roots from the basal tissue were quite common.

The basal enlargements likewise had changed somewhat in character. They had grown considerably and had taken on the rather characteristic deeply convoluted type of growth. These various convolutions were built up for several layers and not infrequently particles of soil were incorporated for some distance within the tissue. This type of growth is illustrated in figure 7.

By the middle and latter parts of the second season both the basal enlargements and the hairy roots had increased markedly in size. In all the specimens examined the large lateral roots which developed as part of the hairy-root overgrowth seemed to have established definite connection with the main stem and to be functioning in a somewhat normal capacity.

Nonparasitic tissue of the wound-overgrowth type not infrequently appeared in association with the basal enlargements of the hairy-root development. This seemed to occur in part as a reaction to the interruption in the downward flow of elaborated food in the stem. Apparently the hairy-root development had something of a wounding or girdling action upon the stem, which resulted in the formation of wound-overgrowth tissue (fig. 8) in combination with the hairy-root tissue. All different stages of combination between wound overgrowth and hairy root seemed to occur. Doubtless in some cases the wound-overgrowth tissue occurred as a result of the girdling action of the hairy-root tissue, whereas in other cases formations which were wound overgrowths at the beginning became infected and hairy-root tissue subsequently developed.

CROWN GALL

Studies of crown gall were made similar to those of hairy root. After 2 weeks the reactions to inoculations were similar to those secured from the inoculations with the hairy-root organism. Frosty hemispherical knobs appeared similar to those of hairy root. However, the knobs on crown gall were not observed to develop further. After 3 weeks the lateral extension of the crown gall was about 1 mm greater than that of hairy root and the surface was lobed and comparatively smooth. After 4 weeks the crown galls had increased considerably in size and often showed a lateral extension of 5 to 8 mm. No root primordia were observed.

After longer periods the basic characters of the crown galls did not change, although the formations increased greatly in size as the weeks passed. No roots have been found growing directly from the crowngall tissue and only infrequently from the main stems of the Yellow Transparent apples near the gall tissue. The sequence of development of the crown galls is shown in figure 8, *F*, *G*. The influence of temperature and moisture on the development of crown gall under controlled conditions has been reported by Riker (7).

MIXTURES OF CROWN GALL AND HAIRY ROOT

Parallel inoculations were made with mixtures of the crown-gall and hairy-root bacteria. The resulting overgrowths developed along the lines previously mentioned for crown gall and hairy root and showed all gradations between those two types of formations. The time of the growing season seemed to exert some influence in determining which type of development would appear first and predominate. The ascendancy of the hairy-root organism was greatest com-

paratively early or late in the growing season, when the temperature was somewhat low. On the other hand, in approximately the middle of the growing season, when the temperature was somewhat high, the crown-gall organism appeared to have the advantage.

Wound and Callus Overgrowths

The control wounds that were made in conjunction with the inoculations for crown gall and hairy root produced only slight reactions. For the first 2 weeks there was practically no difference between the control injuries and those inoculated with the two organisms (fig. 8). However, after 3 weeks the callus formations which appeared began to decrease in size as the process of healing progressed; by the fourth week the soft outer tissue had begun to slough off; after 5 or 6 weeks the characteristic wound tissue had formed and the development of these wound reactions ceased. Natural infection of the control wounds was rare.

Parallel studies were made upon wound overgrowths induced by girdling with wire. As already stated, aluminum-alloy wire was wrapped twice around the stem and then fastened in order to determine what the reaction of the host plant would be to this interruption in the downward passage of elaborated food. After 1 week there was practically no change, but after 2 weeks the wire wrapping was very tight about the bark (fig. 8, *B, C*). After 3 weeks it seemed to be cutting into the bark tissue. After 4 weeks a swelling appeared above the wire that in some cases was sufficient almost to hide it from view. After 5 weeks this wound tissue had increased to a lateral extension of 2 to 4 mm and the wire was almost completely hidden. After 6 weeks the growth had increased in size and after 8 weeks it had a lateral extension of approximately 7 mm around most of the stem. These nonparasitic overgrowth developments so increased in size as the season progressed that at the end of the growing season their diameter was several times that of the main stem. The surface of this tissue had more or less broad undulations very different from the comparatively smooth lobes of the crown-gall tissue or the deep convolutions of the hairy-root tissue. There was a definite cortex. This type of tissue resembled more closely the tissue of hairy root than that of crown gall. As already stated, complete intergradations were found between the surface characters of this type of development and those of hairy root.

Similar wound overgrowths developed after certain knife injuries made on the underground parts of apple stems, about an inch above the union, by an upward cut through one third to one half of the diameter of the stem. A small piece of adhesive tape was inserted under the tissue thus cut in order to prevent its direct healing and to simulate conditions at the lower ends of scions that failed at the tips to make union with the roots. Trees without the tape barriers in the cut usually healed normally in a few weeks without the development of excessive callus. In trees having tape barriers, a small callus had formed on the lower tip of the cut tissue after 1 week (fig. 8, *D*). Within 2 weeks the callus had grown considerably and after 3 weeks it had a lateral extension of several millimeters. The increase in size continued until characters of wound overgrowth appeared similar to those induced by wire girdles. At the end of the season overgrowths of this type had a lateral extension from 1 to 3 times greater than the

diameter of the main stem and showed the characters of wound overgrowth as already described.

These studies on various overgrowths of known origin were very helpful in the diagnoses of overgrowths that developed under natural conditions.

DATE OF NATURAL INFECTION

The natural occurrence of the various overgrowths in this graft-knot complex was recorded at stated intervals throughout three growing seasons. No natural occurrence of crown gall was found among the trees under observation. In other plantings in this locality where a large number of trees were observed, approximately 0.1 percent of the trees were affected with crown gall. The early stages of excess callus or wound overgrowths appeared from time to time. Some of these were grown over as the trees developed (*12*), and some, like the girdle or wound knots (fig. 8), continued to develop; but in this particular locality most of them sooner or later became infected by hairy-root bacteria, and their classification was changed to hairy root. Hildebrand (*4*) has recorded the activity of root-chewing insects in opening infection courts in such tissue. Since the location of this work was made largely on the basis of the severity of the hairy-root infection, this result was expected.

The natural occurrence of hairy root in 1929 on first-year apple trees grown from string-wrapped grafts was recorded at weekly intervals (fig. 1). Natural infection appeared slowly until the end of June, when 11 percent of the trees showed symptoms of disease. By the end of July practically no increase had occurred. However, by the end of August, 28 percent of the trees showed infection; and by the end of September, 38 percent. After this time, apparently correlated with lower temperature and perhaps cessation of growth by the host plant, there was comparatively little increase until the return of warm weather. During the second year (1930), on the same trees (fig. 2), there was a progressive increase up to 49 percent in November, when the trees were removed.

In 1930 the natural occurrence of hairy root on first-year trees (fig. 2) grown from string-wrapped grafts was quite similar to that in 1929. There was 4 percent infection early in July, 13 percent early in August, and 27 percent early in September. There was relatively little increase between that time and the middle of November, when there was 30 percent. During the following year (1931; fig. 3) on the same trees, that were second-year trees by this time, there was practically no development until June. By early July there was an increase to 37 percent, by early August to 42 percent, by early September to 48 percent, and by late October to 57 percent.

In 1931 the natural occurrence of hairy root on first-year trees (fig. 3) grown from string-wrapped grafts was very similar to that of the previous year. Only 3 percent appeared early in July. Early in August there was 14 percent; early in September, 26 percent; and late in October, 38 percent.

Similar results were secured for tape-wrapped grafts (fig. 5). However, the appearance of symptoms was delayed somewhat by the tape wrapping.

The natural occurrence of hairy root in 1930 on trees grown from tape-wrapped grafts was recorded as before. The first hairy-root symptoms appeared early in July, when 2 percent of the trees showed

hairy root.   Early in August, 9 percent showed hairy root; early in
September, 25 percent; and late in September, 30 percent.   The next
year (1931) the number of trees infected rose gradually to 50 percent.
   In 1931 the natural occurrence of hairy root on tape-wrapped grafts
began with 6 percent early in August.   Early in September there was
18 percent, and late in October 21 percent.
   The date of natural occurrence of most of the knots on tape-
wrapped grafts appeared to be correlated with that of knots on string-
wrapped grafts.   The date of natural occurrence of knots on both
types of grafts seemed correlated with warm weather, active growth
of the nursery trees, short incubation periods, decreased protection of
the unions by wrappers, and insect activity.
   The average date of natural occurrence of an infection appearing on
any particular date may be determined by subtracting the number of
days of the average incubation period from the date of appearance.
For example, to determine the average date of natural infection for
new developments that appeared in first-season trees on September 3,
1930, one observes (fig. 2) that the average incubation period ending
September 1 began July 21.   Therefore, the new infection showing
September 3 came from infections taking place about July 21.
   The foregoing data indicate that in the plantings under observation
most of the natural occurrence of hairy root was not the result of
infection induced at grafting time, but was due to certain factors that
began to function usually in June after the grafts were planted.

DISCUSSION

   The evidence presented in the preceding pages has a definite bearing
upon the consideration of control measures.   The studies were made
in a place where infectious hairy root was prevalent and consequently
deal with severe rather than with average conditions.   Since nearly
all the bacterial overgrowths on the underground parts of nursery
apple trees occur at the union, it has been commonly considered that
infection takes place approximately at the time the grafts are made.
Various antiseptics are therefore applied to the seedlings and to the
grafts.   Such treatments by the writers have been relatively unsuc-
cessful in this region.   Wrappers of adhesive tape have been less
efficient for control here than in most places.   Apparently, therefore,
the chances of developing control methods would be enhanced by
determining the time and the conditions under which natural infection
takes place.
   From the data presented in figures 1 to 5, only a small part of the
hairy-root infection naturally occurring appeared to be initiated at
grafting time.   However, this small part may be important as a
potential source of inoculum for subsequent infections.
   The date of natural infection for most of the hairy root was appar-
ently correlated with the occurrence of warm weather, rapid growth
of the trees, activity of soil insects, relatively short incubation periods,
and reduced protection from the wrappers employed.   For first-year
trees this date was during the middle and latter part of the growing
season.   New infections continued to occur during the second growing
season.   In other regions, where the trees stood longer in the nursery
row and where hairy root was serious, new infections appeared in the
third and fourth seasons (12).

The possibility of very long incubation periods perhaps needs further consideration. Relatively dormant trees have been found to carry the hairy-root bacteria for long periods and to show no symptoms until active growth was resumed. The problem to be considered is whether small amounts of inoculum at grafting time might remain dormant long enough to account for the occurrence of hairy root late in the first season and perhaps in the second, third, and fourth season. Although this possibility cannot be easily disproved, the following lines of evidence show that if such a condition occurs it is very unusual: (1) Siegler and Piper (*13*) have reported that bacteria inoculated into aerial parts of trees could be reisolated rarely if at all after 65 days; (2) most of the inoculations made at grafting time have yielded results within ordinary incubation periods and the few infections that appeared later were probably caused by natural infection rather than by the inoculations; (3) the studies on incubation periods showed definite maxima beyond which no symptoms appeared; (4) dormant trees that harbored the bacteria after inoculation in the fall showed symptoms promptly, if at all, when active growth was resumed; (5) the phenomena observed are easily explained by other well-defined factors. It appears, therefore, that the evidence at present available does not justify consideration of common incubation periods as continuing long enough after the date of grafting to account for the date on which the greatest part of the natural infection appeared.

Another phase of the complex hairy-root problem which has been at least partly explained is the failure in this locality of the writers' antiseptic treatments and the lower efficiency of adhesive-tape wrappers. Since root-chewing insects seem implicated during the growing season as important factors in opening infection courts, doubtless the present control measures against graft knots may be supplemented by others that take into account the newly determined date of the natural occurrence of infection.

SUMMARY

The seasonal development of crown gall, hairy root, and wound overgrowth has been followed through the growing seasons of 1929, 1930, and 1931 in Kansas. Crown gall and hairy root were induced by inoculation with single-cell cultures of the causal organisms. Wound overgrowth was induced by wire girdles and by knife cuts. Successive stages in the development of all these overgrowths are described and illustrated.

The following records were kept: Air temperature and humidity, soil temperature and moisture, rainfall, development in height and diameter of both first- and second-season nursery apple trees, incubation periods of both the crown-gall and the hairy-root bacteria, occurrence of hairy root following inoculation and under natural conditions, and groups of insects prevalent in the soil. During this study different seasons have been relatively wet, dry, or intermediate.

The incubation periods for crown gall and hairy root have been relatively long in the spring and fall and relatively short in the summer. They were apparently correlated with temperature and with active growth of the trees.

Both crown gall and hairy root developed after suitable incubation periods following inoculations made at grafting time. Adhesive tape wrappers delayed the appearance of symptoms slightly but did not reduce the number of infections which appeared.

Mixtures of various overgrowths appeared, especially on the second-year trees.

The evidence available points to soil insects, including white grubs, wireworms, and fungus gnats, as important agents in opening infection courts for bacteria during the growing season.

Although a small amount of the natural occurrence of hairy root was traced to infection at the time of grafting and was doubtless important as a source of inoculum, most of it seemed to follow natural infection during the middle and latter part of the first growing season and during the second growing season. Much of the natural infection appeared to be correlated with warm weather, active growth of the nursery trees, short incubation periods, decreased protection of the unions by wrappers, and insect activity.

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HAIRY ROOT, CROWN GALL, AND OTHER MALFORMA-TIONS AT THE UNIONS OF PIECE-ROOT-GRAFTED APPLE TREES AND THEIR CONTROL [1]

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INTRODUCTION

The control of hairy root (*Phytomonas rhizogenes* Riker et al.), crown gall (*P. tumefaciens* (Smith and Town.) Bergey et al.), wound overgrowth, and perhaps other enlargements that occur at the unions between scion and root on nursery apple trees grown from piece-root grafts has been attempted in a number of different ways. These attempts have been stimulated by the fact that very commonly the susceptible varieties of nursery trees have shown these enlargements to a serious extent at digging time. In some extreme cases practically a whole planting has been lost. The prevalence of these diseases has been largely responsible for a change in the method of propagation followed by many of the nurserymen east of the Mississippi River. In this region apple trees are propagated commonly by budding, a method which largely obviates the problem of enlargements at the union and which may give a better stand. However, this method of propagation is reported to be more expensive, to require labor during a very busy part of the growing season, and to provide nursery trees on seedling roots. These factors have caused many of the apple-tree growers in the Middle West to continue piece-root grafting despite the loss from these diseases. To find a means of reducing the loss has been the object of the present studies. Several preliminary accounts on certain phases of this work have already appeared (*23, 25, 28, 32, 33*).[2]

ECONOMIC IMPORTANCE

The actual harm done to the nursery apple trees by enlargements at the union has been discussed by several writers, including Stewart (*45*) and Jakovlev (*13*). However, much of the work reported on the injury caused by graft knots [3] is open to question because of the inadequate diagnosis of the malformations studied. As discussed later, the several different kinds of graft knots that occur doubtless produce various effects upon the trees. More evidence is needed on this question of injury. Since the differentiation of certain kinds of graft knots has been accomplished (*18, 19, 26, 27*) the influence of hairy root on the growth of the trees has received some attention. Comparatively little evidence, beyond that already noted by Riker

[2] Reference is made by number (italic) to Literature Cited, p. 937.
[3] A general term for any overgrowths at the union.

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(913)

and Keitt (*31*), has been secured as yet by the present writers on the influence of crown gall and wound overgrowth of known identity on the growth of nursery trees.

Some data on the effect of hairy-root infection on the growth of nursery apple trees were accumulated in 1929 and 1930 (*27*). Second-season trees of the Yellow Transparent variety were employed. Smooth trees and corresponding hairy-root trees that had become naturally infected the previous season were chosen for measurements. On May 17, 1929, the average height and diameter of 100 smooth trees were, respectively, 32.1 and 0.37 inches. Measurements were made of the same trees September 17, 1929. A few of the healthy trees had become infected during the season and were omitted from the final results. The average height and diameter of the smooth trees were, respectively, 51.8 and 0.59 inches; those of the hairy-root trees were 49.2 and 0.56 inches. These averages show a slight advantage in growth during the season, of 2.6 inches in height and 0.03 of an inch in diameter, in favor of the smooth trees. Repetitions of these trials during the season of 1930 also showed a slight advantage, 5.1 inches in height and 0.08 of an inch in diameter, in favor of the smooth trees. These results are in general accord with those of various writers, including Fracker (*6*), Swingle and Morris (*47*), and Muncie (*18*): the last-named reported a reduction in water-flow through woody knots. However, the data presented in the present paper are so limited and the variations among corresponding trees so great that the differences found seem to have little if any significance. These data, however, raise the question whether the hairy root may perhaps be slightly detrimental to the tree.

In an effort to discover whether the hairy roots were able to function as ordinary roots, a rather severe test was made. On May 17, 1929, 100 hairy-root trees that had become infected following inoculation the previous season were treated. The soil was removed so as to expose the main stem of the tree with as little disturbance as possible to the hairy-root development. The stem was then cut off from the main root system just below the hairy-root development, and the soil was replaced. Ninety-five of the trees thus treated lived throughout the season. By September 17, 1929, they had increased, on an average, 4.5 inches in height and 0.05 of an inch in diameter. Similar trials with like numbers of artificially infected trees in 1930 showed almost identical results, only 5 percent of the trees dying. From May 20 to September 20, 1930, the trees increased, on an average, 6.6 inches in height and 0.12 of an inch in diameter. Smooth trees received similar cuts in corresponding positions. This left them with no roots at all, and of course they died promptly. The fact that the trees supported only by hairy-root developments were able to live shows that these roots have certain functional capacities.

The experiments described in the two preceding paragraphs were made on trees that were grown under comparable conditions in adjacent rows in the nursery. It appears, therefore, that trees which were deprived of all roots except the hairy roots made much less growth than untreated smooth trees or trees that had both natural and hairy roots.

In limited trials following pure-culture inoculations Riker and Keitt (*31*) have found crown gall distinctly detrimental to small nursery apple trees.

Whatever may be the actual influence on the apple trees of the various enlargements at the union, it does not lessen the scope of the problem of control. The problem of these enlargements of trees grown from piece-root apple grafts becomes one of prevention.

These malformations vary considerably not only in their external characters but also in their internal structure.

CAUSES AND DESCRIPTIONS OF OVERGROWTHS

Several different kinds of overgrowths occur on nursery apple trees grown from piece-root grafts. Perhaps the most important are: (1) Infectious hairy root, caused by *Phytomonas rhizogenes* (*27*); (2) crown gall caused by *P. tumefaciens* (*10, 43*); and (3) callus or wound overgrowth, which is nonparasitic (*18, 19, 30, 31*). An understanding of the nature of these formations is obviously desirable in making a satisfactory approach to the problem of their control.

Hairy root is the most common enlargement found in some nurseries. It consists usually of a mass of fleshy or fibrous roots that spring from more or less conspicuous enlargements on the main stem. These enlargements seem to have their origin at the union or at some other point of injury through which the bacteria may have gained entrance. The enlargement at the base of the root formation is usually quite hard, owing to irregular masses of woody elements which seem to be directly connected with the vascular system of the main stem. Hairy-root enlargements have been described by various writers, including Hedgcock (*10*), who called them woolly knots to distinguish them from other overgrowths, and recently they have been described in relation to *Phytomonas rhizogenes* by Riker et al. (*27*). These parasitic hairy-root developments should not be confused with other hairy-root formations which apparently are nonparasitic (*18, 24, 31*).

Crown gall occurs as a result of infection by *Phytomonas tumefaciens*. The causal bacteria may supposedly gain entrance to the tissue at the time the graft is made, or through wounds produced at some later period (*18, 31, 38, 41*). The galls proper on apple are comparatively smooth and as a rule do not have roots growing directly from the surface, although roots frequently grow from the main stem around the edge of the gall. Ordinarily the surface of these galls is not covered with a true bark but either with a layer of dead cells or with actively growing gall tissue. The interior is usually soft like cortical tissue but may contain hard woody elements. Galls of this description render infected trees unsalable, but the small percentage of such galls on nursery apple trees in the Middle West makes them of little economic importance. A more complete description of these crown galls has been given by several writers, including Smith et al. (*43*), Riker and Keitt (*31*), and Muncie (*18*).

Callus or wound-overgrowth development begins commonly on the cut surfaces of the scion and root after they have been fitted together and stored for some time in moist packing material. This formation of callus is of course necessary to establish a union between the scion and the root. However, a union may be imperfect because of a variety of influences, such as a poor fit in grafting, a scion larger than the root, loose wrapping, irregular callus development, and the formation of cork over the callus tissue (*10, 18, 31, 35*). In many

cases the development of callus may continue over a long period and may result in an enlargement of sufficient size to deform the tree. Enlargements of this character have been stimulated by girdling young trees with wire or with a wrapper that failed to rot in a comparatively short time (*24, 29, 30*). They are caused apparently by the blocking of the downward flow of elaborated food by the girdle and the activity of the tissue immediately above the point of restriction. Apparently a similar condition occurs where the union between the scion and the root is relatively imperfect. As explained later, it has appeared from the cause of these malformations that they might be controlled, as pointed out by Hedgcock (*10*), if a better union between scion and root could be secured and the formation of excess callus prevented by carefully fitting the grafted parts and by using suitable wrapping.

Mixtures of these different types of development occur with more or less frequency from time to time. Complete intergradations between them may be found in many nurseries. Likewise, inoculations with mixtures of the crown-gall and hairy-root organisms have given complete intergradations of the crown-gall and hairy-root symptoms (*24*). Perhaps the most common mixture encountered in the average nursery is that of hairy-root and wound overgrowth. These occur either from the infection of wound overgrowths with hairy root or from the formation of overgrowths at hairy-root infections, doubtless in large part because of the same factors that induce overgrowths above mechanical injuries or girdles. The relative frequency of different types of mixtures has been found to vary in different localities.

Although hairy root, crown gall, and wound overgrowths are apparently the more important types of graft knots, other kinds of overgrowths may occur with considerable frequency under certain conditions. Among these may be mentioned (1) burrknots, described by a number of writers, including Birmingham (*1*), Brown (*2*), Swingle (*46*), Hatton et al. (*9*), Carne (*3*), and Siegler and Piper (*40*), the last-named workers producing malformations morphologically identical with their apple strain of bacteria but failing to isolate these bacteria from naturally occurring burrknots; (2) incompatible unions, mentioned by Riker (*22*); and (3) noninfectious hairy root described by Muncie (*18*), Riker et al. (*27*), and others, the cause of which still remains obscure.

Since the relative prevalence of the different kinds of graft knots has a definite bearing upon the types of control measures that should be employed, their distribution in the nursery was examined.

DISTRIBUTION OF OVERGROWTHS IN THE NURSERY

Malformations, particularly at the unions, on trees grown from piece-root apple grafts appear to occur wherever apple trees are propagated by this method. There has been some confusion about the relative distribution of these malformations because of the difficulty of diagnosis. It is only within the last few years that the causal differences in hairy root, crown gall, and callus developments have been noted. In the survey reported by Riker and Keitt (*31*) crown gall was separated from wound overgrowth and hairy root. However, for the most part these writers placed both wound over-

growths and hairy root in the same classification without distinction. Muncie and Suit (*19*) have reported the results of extensive surveys in which they found that infectious hairy root was of comparatively small consequence. In the light of other investigations (*25, 27, 37*) where the cause and symptoms of infectious hairy root are more clearly defined, it appears that this disease is of great economic importance, especially under some conditions.

The distribution in the nursery of the various malformations under consideration on the underground parts of nursery apple trees has been studied by the present writers in several different localities. Each tree in the nursery row was examined after being dug, and the relative positions of the smooth and the knotted trees were noted. If an overgrowth was found, its size, position, and character were recorded in detail. Two examples of very common types of occurrence and distribution are given in figures 1 and 2. The actual field data have been so arranged that much about the condition of each tree might be indicated by two letters. A comparatively light incidence of overgrowths often shows the type of distribution illustrated in figure 1. This record was taken in one long continuous row of which the trees recorded comprise only a small part. A common distribution showing a heavy incidence of overgrowths appears in figure 2. Results similar to those just cited (figs. 1 and 2) were secured in most of the studies on control reported later (tables 1 to 5). The details of these records are omitted because of their large volume.

These records, of which figures 1 and 2 are small and condensed examples, suggest that (1) if the hairy-root organism comes from the soil where the grafts are planted it is well distributed there; (2) if it comes in with the grafts it is also well distributed among the grafts; and (3) after the disease develops it may spread somewhat along the nursery row. The question of the source of hairy-root inoculum has received detailed consideration in other papers (*11, 29*). Crown gall was found only occasionally. In most of the studies reported in this paper it comprised less than 1 percent of the overgrowths. Excess callus or wound overgrowths occurred with relative frequency on the first-year trees; on second-year trees they were often either healed over or infected with hairy-root bacteria; on still older trees they frequently occurred in combination with hairy root.

DISTRIBUTION OF OVERGROWTHS ON TREES

Most of the overgrowths on the underground parts of the main stems of the trees occur at the union between scion and root, as shown in figures 1 and 2. Further records of 54 trials on trees grown from grafts wrapped with string and of 54 corresponding trials on trees wrapped with adhesive tape are shown in table 1. These trials were made in Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, and Wisconsin on 1- to 4-year-old trees of the following varieties: Bayfield, Black Ben, Delicious, Dudley, Early Harvest, Fameuse, Florence (crab), Gano, Golden Winesap, Goodhue, Jonathan, McIntosh, Northwestern Greening, Oldenburg, Perkins, Red Siberian (crab), Red Wing, Wealthy, Whitney (crab), Yellow Transparent, and York Imperial.

On an average, less than 5 percent of the trees had overgrowths on the scions and less than 2 percent on the roots, whereas approximately 25 percent of the trees grown from commercial string-wrapped grafts, and from 7 to 14 percent of the tape-wrapped trees, had graft knots at the unions. There was considerable variation in individual trials.

```
o   o   o   o   o   o   o   o   o   o   Ks  Hu  o   o   o
o   o   o   o   o   o   o   o   o   Hu  o   o   o   o   o
o   o   o   Hu  o   o   o   o   o   o   Hu  o   o   o   Hu
o   o   o   o   o   o   o   o   o   o   o   o   o   o   o
Ku  o   o   Hu  hu  o   o   o   o   o   o   hc  o   o   o
o   o   o   o   o   Hu  o   kc  o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   o   o   Hc  o   o   o
hc  Ks  o   o   Hc  o   o   o   o   o   o   Hc  o   o   o
o   o   Hc  o   o   o   o   o   o   o   o   o   o   Hc  o
o   Ku  Ks  o   o   o   o   o   o   Hu  o   o   o   o   o
Ku  o   o   o   hu  o   o   o   o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   o   o   o   o   o   o
o   Ku  o   o   o   o   o   o   o   o   o   o   Ku  o   o
o   o   Hu  o   o   o   o   Hu  o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   o   o   o   o   o   o
o   o   o   o   Hc  o   o   Hu  o   o   Hu  o   o   o   o
Hu  o   o   o   o   o   o   o   o   o   o   Hu  o   o   o
o   o   o   o   o   o   o   o   o   Gs  o   o   o   o   o
o   o   o   o   o   o   Hu  Ku  o   o   o   o   o   o   o
o   o   o   o   o   o   o   o   o   Hu  o   o   o   o   o
o   o   o   Hc  o   o   o   o   o   o   o   Hu  Hc  o   Hu
o   o   o   o   o   o   o   o   o   o   o   o   Hu  hc  o
o   o   o   o   Hu  o   o   Ku  o   o   Hu  o   Hu  o   o
Hu  o   o   o   o   o   o   o   o
```

FIGURE 1.—Distribution of enlargements in one continuous row (reading from left to right) on 2-year-old Yellow Transparent apple trees propagated in Iowa from tongue grafts. The characters of the individual trees are summarized according to the following symbols: *, Smooth; G, large crown gall; H, large hairy root; h, small hairy root; K, large callus or wound overgrowth; k, small callus or wound overgrowth; c, located on the scion; u, located on the union; s, located on the stock.

From 0 to 18 percent of the trees had overgrowths on scions, from 0 to 93 percent on unions, and from 0 to 7 percent on roots. These results confirm those of a number of earlier papers. However, the percentages of overgrowths at the union in these trials were lower than those reported by several workers, including Doidge (4) and Muncie (18). This difference is probably due to recent improvements in grafting technic and in cultural practices.

Some difficulty was experienced in differentiating incipient hairy root from burrknots on the scions of such varieties as Bayfield and

Okabena, on which burrknots occur frequently. Such burrknots have not been included in these records. Overgrowths were found from time to time on the lateral roots. In some cases they appeared to follow mechancial injury incident to cultivation, but more frequently they were in positions where only insects could have reached them. This subject is discussed more fully by Hildebrand (*11*).

Hu Hu Hu ○ *Hu Hu Ks* ○ *Hu* ○ *Hu* ○ *Hu Hu* ○
○ ○ *Hu* ○ ○ *Hu hc Hu Hu* ○ *Hu Hu Hu Hu* ○
○ *Hu Hu Hu* ○ *Hu* ○ *Hu* ○ *Hu* ○ *Hu* ○ *Hu Hu*
Kc ○ *Hu* ○ *Hu Hu Hu Hu* ○ ○ *Kc Hu* ○ ○ *Hu*
Hu ○ *Hu* ○ *Hu Hu* ○ *Hu Hu Hu* ○ ○ ○ *Hu Hu*
Hu Hu Hu Hu Hu Hu ○ ○ *ku* ○ ○ *kc kc Hu* ○
○ *Hu* ○ *Hu* ○ *Hu Ku Hu Hu Hu Hu* ○ *Hu* ○ ○
Hu ○ *Hu Hu* ○ ○ *Hu* ○ *Hu* ○ *Hu* ○ *Ku Hu* ○
Hu ○ *Hu Hu Ku Hu Hu* ○ *Hu Hu* ○ ○ ○ *Kc Ku*
Hu ○ *Hc Hu* ○ *Hu Hu* ○ *Hu* ○ *Hu* ○ *Hu* ○ ○
Hu Hu Hu ○ *Hu Hu* ○ *Hu* ○ *Hu Hu* ○ *Hu* ○ *Ku*
Hu hc ○ *Hu* ○ *Hu Hu* ○ *Hu* ○ ○ *Hu Hu Ks Ku*
Hu ○ *Hu Hu hu Hu* ○ ○ ○ *Hu Hu Hu* ○ *Hc Hu*
○ *Hu Hs Hs* ○ ○ *Hs* ○ *Hu Hu Hu* ○ *Hu Hu Hu*
Hu Hu ○ *Hu Hu Hu Hu Hu Hu hu Hu Hu* ○ *Hu Hu*
Hu Hu Hu Hu ○ *Hu Hu* ○ *Hu* ○ *Hu* ○ *Hu Hu* ○
Hu Hu ○ ○ *Hu Hu Hu* ○ *Ks* ○ *Hu* ○ ○ *kc* ○
○ *hu hu hu Kc Hu* ○ *Hu Hu Hu Hu Hu Hu Hu* ○
○ ○ *hu* ○ *hc* ○ ○ *c Hu Hu Hu Hu hc Hu Hu*
Hu Hu ○ *Hu Hu Hu* ○ *Hu* ○ *Hu* ○ ○ *Hu Hu Hu*
○ ○ ○ *kc Hu Hu Hu Hu Hu* ○ ○ *Hu Hu Hu Hu*
Hu Hu ○ ○ ○ *Hs Hu Hu hu* ○ *Ku Hu* ○ *Ku* ○
Hu Hu Hu Hu ○ *Hu hu Hu*

FIGURE 2.—Distribution of enlargements in one continuous row (reading from left to right) on 2-year-old Wealthy apple trees propagated in Kansas from tongue grafts. The characters of the individual trees are summarized according to the symbols shown for figure 1.

TABLE 1.—*Relative frequency of occurrence of enlargements on various underground parts of nursery apple trees grown from piece-root grafts, 1929–31*

Year	Trials	Wrapper	Trees examined	Trees with enlargements on—		
				Scion	Union	Stock
	Number		*Number*	*Percent*	*Percent*	*Percent*
1929	18	String	8,469	4.6	24.8	0.8
	18	Tape	11,848	3.0	7.2	1.1
1930	24	String	8,504	1.1	25.1	1.0
	24	Tape	8,760	1.1	14.2	1.8
1931	12	String	4,132	3.2	25.0	.5
	12	Tape	4,984	3.1	13.6	1.3

CONTROL OF OVERGROWTHS

METHODS AND MATERIALS

In experiments for the control of the various overgrowths on the unions of piece-root-grafted nursery apple trees, four factors have been considered: (1) The kind of seedling employed, (2) the treatment of seedling roots with various antiseptics, (3) the manner of making the graft, and (4) the material used for wrapping the graft. As is pointed out later in this paper and in more detail by Hildebrand (*11*) and Riker and Hildebrand (*29*), wounds produced by soil insects represent another factor deserving special consideration.

The practice of excision of overgrowths and subsequent antiseptic treatment is not considered in the present paper, because of previous unpromising results.

A considerable range of conditions was sought in making the different trials. For this reason tests were conducted in representative nurseries in a number of States, namely, Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, and Wisconsin. The complexity of the problem and the multiplicity of factors involved have hindered the rapid progress of the work and must be considered in the interpretation of the results secured. Various factors have operated alone or in combination in different nurseries during the same season and in the same nursery in different seasons. Certain control measures successful in one place have failed completely in another. Consequently, corresponding variability has been introduced into repetitions of the trials, the more important trials having been repeated in several places in the same season and in the same place in different seasons. The trial grafts differed from the corresponding control grafts in only one particular.

The varieties of trees employed differed in different localities. In general, varieties grown in comparatively large numbers and those reported to be susceptible to enlargements at the union were chosen for the tests, but some of the less popular and less susceptible varieties were used also.

The age of the trees used differed in different localities. In the Northern States the trees stood 3 or 4 years in the nursery; in the Southern States they were lifted after 1 or 2 growing seasons. In a number of cases trees grown only 1 season were examined in order to secure a preliminary idea of the efficiency of the methods being employed. The examination was made by removing the soil from around the tree until the union could be seen. When this was done, special care was taken not to injure the trees, and after the examination the soil was replaced. Because of the labor involved, especially during wet and cold weather, only a small number of trees were examined, usually 100 receiving the same treatment and 100 controls; in some cases only 50 of each. These examinations were made at random in the larger plantings.

Most of the records of the different trials were kept in considerable detail. The presence or absence of an overgrowth on the underground parts of each tree was noted. Records were kept of the location, relative size, probable cause, and character of tissue of all the enlargements and of the presence, number, character, and size of hairy roots. However, in a number of cases only a summary of the condition at the union was recorded, especially when a representative

distribution had been secured for a variety in a given locality and when the numbers involved were large.

Because of the great volume of the records only synopses of the data are represented here, except in one representative instance.

RELATION OF TREES TO CONTROL FACTORS

The age of the nursery apple trees has an important bearing upon the significance of the results secured. In accord with the results of Melhus and Maney (*16*), the percentage of graft knots on 1-year-old trees was found to be of doubtful value in predicting the percentage on the same trees in later years, but is important in showing the efficiency of control measures applied at grafting time. A comparison of the results with 1-year-old trees and the same trees when 2 years old shows that important factors continue to operate in the second season.

Comparisons between the percentages of smooth unions on first- and second-season trees, some of which had been wrapped with tape and others with string, are shown in table 2. The 10 trials involved records of data on 14,435 trees. The observations on first-season trees were made by direct examination after the soil had been removed from the unions. The soil was then replaced. Because of the labor involved, a comparatively small number of trees in each lot (between 50 and 100 taken at random through the entire planting) were examined.

TABLE 2.—*Percentages of smooth unions on grafts of various plantings of nursery apple trees at different ages*

State	Variety	Amount of graft knot	Tape				String			
			First season	Second season	Third season	Fourth season	First season	Second season	Third season	Fourth season
			Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
Kansas	Wealthy	Great	96	77			54	37		
	do	do	86	67			34	39		
Minnesota	do	do	100	94			46	48		
	Okabena	do	100	58			16	4		
	do	Small	95	99			55	74		
	Oldenburg	do	100	98			68	74		
Iowa	do	do	92	94			56	79		
	Yellow transparent	do	100	97			84	89		
	do	do	91	96			82	88		
	Wealthy	do	97	90			84	86		
Nebraska	do	do	96		81		70		81	
	do	do	100		94		92		82	
	Bayfield	Great	84			49	48			18
	do	do	82			36	58			29
Minnesota	Wealthy	do	92			59	40			14
	do	do	100			67	46			34
	Okabena	do	84			44	36			13
	do	do	100			42	16			8

When the amount of graft knot was large, several results were noted which deserve comment. Infectious hairy root was the predominant type of overgrowth. The trees wrapped with tape generally appeared to be protected during the first year against the several factors causing the development of graft knots, but this protection had

little influence during the second year when the agencies producing graft knot continued to operate. The question as to just what these agencies were and the method by which they acted has been discussed in more detail by Riker and Hildebrand (*29*). These writers have also reported that the possibility of long incubation periods for bacteria on actively growing trees under favorable conditions seems too remote to have much bearing upon this question. Siegler and Piper (*40*) have reported that causal bacteria could be isolated rarely if at all from aerial crown-gall inoculations after 65 days.

When the amount of graft knot was relatively small it appeared that many of the unions wrapped with string which had shown wound overgrowths or excess callus by the end of the first year became smooth by the end of the second year. This process had been noticed earlier by Melhus and Maney (*16*), with respect to poor unions. The time required for excess callus to become smooth depended upon the size of the callus, the vigor of the tree, and the absence of other knot-producing agencies. No cases were noted of recovery from infectious types of overgrowths.

Comparisons between the percentages of smooth unions on trees of different ages, e.g., on first- and third-season trees and on first- and fourth-season trees, gave similar results. A total of 8,906 trees was examined in these trials.

These results have a definite bearing upon the interpretation of the various control studies. It appeared quite clear that the first season is not necessarily the most important for the development of graft knots; however, it may be the most important for interpreting the results of the control measures applied at grafting time. When the amount of graft knot was large and the graft knots were predominantly of the infectious hairy-root type, the first season was often less important than the seasons that followed. On the other hand, when the amount of graft knot was small and the graft knots were mostly of the wound-overgrowth type, there was often no material change from the first season. Except during an epidemic of hairy root, the percentages of smooth unions following string wrapping increased during the second season; however, they never reached the point where they exceeded the percentages of smooth unions following tape wrapping. No epidemics of crown gall were observed. Since control measures thus far have been applied almost entirely at grafting time and during the first season, these studies show that under certain conditions the methods used cannot be expected to yield fully satisfactory results. However, they can, perhaps, reduce the intensity of an epidemic by lowering the percentages of trees that provide inoculum for later years. Thus far there is no evidence to show that the crown-gall (*31*) and hairy-root (*11*) bacteria may gain entrance into the host tissue in any way except through wounds. Consequently, these studies point to the root-chewing insects in the soil and to injuries during cultivation as agents in the production of epidemics, certainly after the first season and probably during the first season.

INFLUENCE OF SEEDLING STOCK USED

To determine what influence the seedling stock might exert on the number of enlargements that develop from piece-root grafts, 32 trials were made involving 31,000 grafts. Seedlings from France and from

the States of Colorado, Kansas, Vermont, and Washington were compared. The trials were made during several years, in Iowa, Kansas, Missouri, Nebraska, and Oklahoma, with scions of the following varieties: Rome Beauty (dark red sport), Delicious, Golden Delicious, McIntosh, and Wealthy. The details of these studies are omitted because of their volume.

In all but nine of the individual trials the percentage differences of smooth trees on grafts with different seedlings were relatively small. When the percentage differences were 10 or more the values are listed as examples to show the variations. In these trials the percentage differences in smooth trees obtained from the use of different lots of seedling stock were 18, 32, 12, 35, 32, 12, 10, 11, and 11. The first five of these examples showed a predominance of infectious hairy root. In these cases it seems clear that in each trial one lot of the seedlings employed carried the hairy-root organism on the surface to a much greater extent than the other. Out of the 64 lots of seedlings employed in the 32 trials, 39 lots yielded over 80 percent of smooth trees when they were lifted. The relatively large percentage of smooth trees and the diversity in type of the enlargements that were found suggest that hairy-root bacteria on the surface of seedling roots was not a very serious factor in a majority of the cases studied. Although different lots of seedlings showed considerable variability, no correlation in the amount of graft knot was found between one year and the next with the seedlings from one region or even from one nursery. But in several cases it was found that various shipments of seedlings from a particular nursery gave consistently unfavorable results in several different States in the same year. However, the next year the seedlings from that same nursery were among the best employed. To summarize these results, it appears that, in accord with the results of Waite and Siegler (*49*) and Siegler and Piper (*41*), circumstantial evidence was found indicating that the causal bacteria may be carried on the surface of certain lots of apple seedlings. It therefore seemed desirable to try isolations from the surfaces of suspected seedlings.

Out of 19 attempts at isolation, positive results were secured in 15; repetitions of these trials gave similar results. The hairy-root bacteria were obtained from seedling apple roots in 24 out of 26 trials. The identity of the bacteria was determined by certain cultural characters and by successful inoculations into plants.

Other factors besides the presence of hairy-root bacteria should be mentioned in considering the incidence of overgrowths in grafts made with different lots of seedlings. The physiology of the seedling in relation to ripeness and to resistance is well worthy of further study. Attention has been given by several writers (*8, 11, 34, 42*) to the question of resistance of different varieties of fruit trees to crown gall and related diseases. It therefore seemed desirable to determine whether apple seed from different sources would produce seedlings that differed in resistance to graft knots.

The exact source of the seed was not easy to determine. However, in a few instances the seed was followed from the variety of apple that produced it, and in three cases the seed was traced to individual trees. Two experiments with seed from Hopa (crab) and Meader trees may be mentioned together. The special seed was planted in the same field with commercial seed, and the seedlings were used to make piece-

root grafts with McIntosh scions. At the end of two growing seasons from the time the grafts were planted, data were recorded for 500 trees from each seed source. The trees grafted on seedlings grown from Hopa seed were 89 percent smooth; those from Meader seed also were 89 percent smooth. Those from four corresponding lots of commercial seed were, respectively, 58, 56, 56, and 61 percent smooth. In a similar trial involving similar numbers, seed from Wealthy trees was employed to grow seedlings on which were grafted Jonathan scions. The trees grafted on seedlings grown from Wealthy seed were 91 percent smooth. Those from three corresponding lots of commercial seed yielded, respectively, 74, 78, and 78 percent smooth trees. Although these results are too few to justify conclusions, they are sufficiently striking to show the desirability of further work in this direction. Such work presents the difficulty, however, of differentiating between the amount of true resistance and the amount of response to causal bacteria carried on the surface or within small injuries. Although these Hopa, Meader, and Wealthy seedlings received the same treatment as the commercial seedlings, the possibility remains that they may merely have escaped surface contamination by the hairy-root bacteria.

VALUE OF CERTAIN ANTISEPTICS

The presence of the hairy-root bacteria on the surfaces of certain lots of seedlings points to the possible value of antiseptic treatments for seedling roots.

Certain antiseptics appear to have promise at times in preventing infection when the grafts are made from seedlings carrying undesirable bacteria. Since Waite's work in 1909,[4] antiseptics have been employed with varying success by a number of workers (*7, 10, 12, 15, 16, 20, 31, 36, 44, 48, 49,* and *50*). The present writers made trials with a considerable number of antiseptics in the hope of controlling the various overgrowths due to bacteria that gain entrance into the plant at grafting time. However, since it has been determined, as explained earlier, (1) that not all graft knots are due to bacteria, (2) that not all seedlings carry infectious bacteria in significant numbers, and (3) that a considerable percentage of the graft knots are caused by hairy-root bacteria that gain entrance to the tissue months after the union is established (*11, 29*), it is not surprising that the results of work with antiseptics have failed to be uniformly promising.

A considerable number of substances were given laboratory, greenhouse, and field trials. The best of these were selected for more extensive trials. Thirty-five treatments, with corresponding controls, were made in the preparation of approximately 25,000 grafts in Iowa, Kansas, Minnesota, Missouri, Oklahoma, and Wisconsin with scions of the following varieties: Rome Beauty (dark red sport), Jonathan, Stayman Winesap, Wealthy, and Yellow Transparent. Since most of the antiseptics were not found to be satisfactory, mention is made of only three.

Hydroxymercurichlorophenol sulphate (Semesan) was used in 15 trials, which are summarized in table 3.

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[4] It appears that Waite was the first to employ an antiseptic (formaldehyde) solution as a dip for apple stocks and scions before grafting. Although this early work was unpublished it has already been noted (*49*).

TABLE 3.—*Effect of three antiseptics on occurrence of malformations at the unions of piece-root-grafted nursery apple trees*

Antiseptic	Trials	Age of trees	Wrapper	Total trees examined	Stand	Trees showing indicated condition at union		
						Smooth	Small knot [a]	Large knot [b]
	Number	*Years*		*Number*	*Percent*	*Percent*	*Percent*	*Percent*
Semesan	10	1–3	String	2,172	52	74	8	18
Controls	10	1–3	do	2,666	52	70	10	20
Semesan	5	1–2	Tape	1,675	66	85	1	14
Controls	5	1–2	do	2,550	63	81	1	18
Silver nitrate	6	1–3	String	2,150	67	86	4	10
Controls	6	1–3	do	2,676	53	82	6	12
Silver nitrate	8	1–3	Tape	4,098	54	95	2	3
Controls	8	1–3	do	4,204	66	94	1	5
Mercuric chloride	2	2	do	834	64	94	1	5
Controls	2	2	do	1,792	64	80	0	20

[a] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.
[b] This class included all enlargements larger in cross measurements than half the diameter of the tree.

In 10 of the trials the grafts were wrapped with string or cloth and treated in a 1:400 solution according to the directions of Waite and Siegler (*49*). In the 5 other trials only the seedlings were treated and the grafts were wrapped with adhesive tape. The control grafts received no treatment whatever. In one trial in Minnesota, after 1 season of growth with a string wrapper, the treated grafts showed 68 percent smooth, whereas the untreated grafts showed 42 percent smooth. In another trial, in Missouri, after 2 seasons of growth with a cloth wrapper, the treated grafts showed 56 percent smooth and the untreated grafts 88 percent smooth. The Minnesota trial showed a difference of 26 percent in favor of the treatment; whereas the Missouri trial showed a difference of 32 percent against the treatment— great and contradictory differences. However, in most cases the differences were rather small and more of them were in favor of the treatment than against it. The averages given in table 3 show several percent in favor of the treatment. The average differences are considered to be within the range of experimental error. The stand appeared not to be influenced by the treatment. These results appear in general accord with the following statement by Siegler (*39*): "We experimented with the use of Semesan which gave good results in earlier years but which recently has not proved so efficacious." As explained earlier, the factors that operate to induce overgrowths in one place may be very different from those in another. The case in which 32 percent more enlargements were induced in the treated grafts than in the controls was very puzzling. An explanation was sought in a laboratory study of this substance.

The efficiency of the hydroxymercurichlorophenol sulphate was determined against the crown-gall organism with a modified Rideal-Walker technic as used by Keitt, Shaw, and Riker (*14*). In the absence of any organic matter a dilution of 1 in 800 at 20° C. killed a measured concentration of a 48-hour-old washed culture of the bacteria in 10 minutes, but not in 5 minutes. As was to be expected, the presence of a little soil extract or other organic material considerably reduced the efficiency of this antiseptic. It seems likely that, under nursery conditions, enough soil might easily be present to counteract

the germicidal effect of the chemical. Under such conditions it appears probable that the treatment might spread rather than destroy any bacteria present.

Silver nitrate, 1:1,000, gave rather poor results in the control of overgrowths. The seedling roots were dipped in this substance and held for 2 minutes; no subsequent treatment was made. In 6 trials the grafts were wrapped with string and in 8 with tape. A summary of these trials is included in table 3. The differences between the percentages of smooth trees resulting from the treated grafts and those resulting from the untreated grafts somewhat favor the treatment but are still within experimental error. However, the injury from the silver nitrate produced a noticeable reduction in stand.

The statistical method of Fisher (5) was applied to these data, in consultation with Dr. R. A. Brink, of the University of Wisconsin, in order to see whether the treatments might be more effective than they appeared. The significance of the mean difference in relation to the standard deviation of the difference was calculated. For treatments with hydroxymercurichlorophenol sulphate and with silver nitrate, the values of P were 0.7 and 0.6, respectively. These calculations, which are omitted because of their large volume, corroborate the conclusion derived from inspection that these treatments had little if any effectiveness in general practice.

Mercuric chloride, 1:1,000, in limited trials has given more promising results than any other antiseptic employed. The average of two trials shown in table 3 indicates that if used in conjunction with tape wrappers it may be effective, especially if the seedlings carry infectious bacteria. Other trials now under way appear thus far to corroborate these results.

Tested by the laboratory method described above, mercuric chloride showed considerably greater toxicity than Semesan. A dilution of 1 in 15,000 at 20° C. killed a measured concentration of a 48-hour-old culture of crown-gall bacteria in 10 minutes, but not in 5 minutes. Further work with antiseptics seems desirable.

COMPARISON OF WELL-MATCHED TONGUE AND WEDGE GRAFTS

The type of graft in relation to the prevention of various overgrowths at the union of piece-root apple grafts was examined in a number of trials. Hedgcock (10) reported that poorly made grafts were more likely than well-made ones to produce callus enlargements and were also likely to become infected. In addition, Riker and Keitt (31) showed that poorly made grafts united only a part of the scion to the root and that a situation similar to a partial girdle resulted. Siegler (38) suggested that the fit of the grafts has been overemphasized. Melhus et al. (17) found that better unions might be secured with wedge grafts. A number of different series of well-fitted tongue and wedge grafts were made. Various lots of these grafts were wrapped with adhesive tape; others were wrapped with string. The results of these tests are presented in condensed form in table 4.

These studies involved 72 different trials, with a total of 27,981 tongue grafts and 21,759 wedge grafts. The results were obtained in 1928 to 1931, inclusive. The experiments were made in Iowa, Kansas, Minnesota, Missouri, Oklahoma, and Wisconsin on the following

varieties: Bayfield, Rome Beauty (dark red sport), Delicious, Dudley, Early Harvest, Golden Delicious, Jonathan, Maiden Blush, Okabena, Oldenburg, Red Wine, Wealthy, Whitney (crab), and Yellow Transparent. The examination of first-year trees, as already explained, was made by removing the soil from around the unions of trees taken at random and not by lifting the trees. The data on comparative lots of 1-year-old trees were taken under one set of conditions, while those on 2-, 3-, and 4-year-old trees were taken under different conditions. Consequently, comparisons are possible between trees of the same age, but not between trees of different ages.

TABLE 4.—*Effect of tongue and wedge grafts on occurrence of malformations at the unions of piece-root-grafted nursery apple trees*

Wrapper	Trials (Number)	Graft	Age of trees	Total trees examined	Stand	Trees showing indicated condition at the union		
						Smooth	Small knot [a]	Large knot [b]
			Years	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
Tape	14	Tongue	1	1,893	69	96	3	1
	14	Wedge	1	1,485	58	93	4	3
	13	Tongue	2	7,716	53	92	1	7
	13	Wedge	2	6,265	65	92	1	7
	7	Tongue	3	3,505	71	89	0	11
	7	Wedge	3	3,072	55	89	0	11
	3	Tongue	4	630	--------	48	4	48
	3	Wedge	4	464	--------	51	5	44
	Summary: 37	Tongue	1–4	13,744	65	89	2	9
	37	Wedge	1–4	11,286	60	89	2	9
String	10	Tongue	1	926	65	59	17	24
	10	Wedge	1	850	57	62	18	20
	14	Tongue	2	8,459	75	74	4	22
	14	Wedge	2	6,681	63	75	3	22
	8	Tongue	3	4,249	71	73	1	26
	8	Wedge	3	2,449	69	72	1	27
	3	Tongue	4	603	--------	24	4	72
	3	Wedge	4	493	--------	15	5	80
	Summary: 35	Tongue	1–4	14,237	70	65	7	28
	35	Wedge	1–4	10,473	61	65	7	28
Tape and string	Summary: 72	Tongue	1–4	27,981	68	78	4	18
-----Do-----	72	Wedge	1–4	21,759	60	78	4	18

[a] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.
[b] This class included all enlargements larger in cross measurements than half the diameter of the tree.

Considerable variations in the percentage of overgrowths at the unions were found in both directions in the comparison of tongue and wedge grafts. These variations occurred whether the grafts were wrapped with string or tape. It appeared from these trials that there was no significant difference between tongue and wedge grafts in the reduction of overgrowths at the union. These results are not necessarily opposed to those of Melhus et al. (*17*), for apparently these writers were concerned primarily with callus developments, whereas the present writers considered all the various kinds of overgrowths discussed earlier.

The reduced average stand of the wedge grafts wrapped with string as compared with that of tongue grafts seemed to have little signifi-

cance because of the great variations in the results and because these variations favored first one and then the other type of graft. Fisher's method (5) was applied to the data on stands of tongue and wedge grafts at the end of the first season, to determine the significance of the mean difference in relation to the standard deviation of the difference. The value of P was 0.4, indicating that the difference had little if any significance. However, it should be noted that wedge grafts wrapped with string were more likely than tongue grafts to come apart before they were planted. Likewise, the wedge grafts had a greater tendency to send up sprouts from the seedling roots. When the grafts were wrapped with tape, the possibility of their coming apart and of the roots sending up sprouts was reduced.

EFFECT OF KIND OF WRAPPING USED

For reducing the percentage of enlargements at the unions of piece-root-grafted nursery apple trees, the kind of wrapping used was the most important single factor studied. A considerable number of different kinds of wrapping were tried, including string, waxed string, chemically treated string, string covered with wax after wrapping, paper, raffia, chemically treated raffia, raffia covered with wax, cloth, waxed cloth, and a number of kinds of adhesive tape. Hedgcock (10) found that, of the wrappers he tested, cloth was the best. In the present trials cloth was found to be better than any of the others except adhesive tape. Consequently detailed reports of the long series of trials in which wrappers other than adhesive tape were employed have been omitted. However, certain determinations were made which deserve mention. It was found that by means of various treatments with different chemicals the string wrappers could be preserved for practically any length of time during the growing season. It was also observed that under average conditions a string wrapper that remained longer than 12 weeks was likely to produce girdling. The time necessary for decay of the wrapper varied considerably under different environmental conditions. When the ground was unusually moist the trees grew more rapidly and the wrapper decayed more quickly. Conversely, when the soil was comparatively dry the wrapper lasted longer, but the trees did not grow so rapidly. The strength of the string decreased rather gradually as the season progressed. This diminution apparently depended on the ready access of soil organisms, for any protected portion of the string retained its strength much longer than the unprotected portions.

The adhesive tape chiefly employed was essentially a cloth wrapping to which had been added a plaster mass. The adhesive-tape wrapper provided several valuable features, including (1) reasonable cost, (2) easy application, (3) firm wrapping, which prevented injury to the union during subsequent manipulations and which facilitated handling, (4) a waterproof covering, which prevented the entrance of undesirable material, (5) a mechanical prevention against the development of excess callus on the surface of the union, and (6) a barrier for some months against insect injury at the union.

Not all kinds of adhesive tape were satisfactory. A small number of trials with electrician's friction tape and with tape spread with "surgeon's mass" gave such unpromising results that they were dis-

continued.   Certain other special tapes in which various chemicals were incorporated sometimes showed no advantage over ordinary tape.   Some of these special tapes had serious disadvantages; a few of them considerably reduced the stands, and two samples that were tried prevented any of the grafts from growing.   Several of these special tapes were comparatively slow to decay in the soil and for that reason might last long enough under some conditions to produce girdling.   The adhesive tape [5] employed in these trials was rather similar to that used extensively by the medical profession, but with modifications in the interests of economy.

The manner of application of the adhesive tape was found to be important both in relation to speed and to the results secured.   Scions 5 to 6 inches long and roots 3 to 4 inches long were used for the grafts, which were well made from good materials.   The method of applying the adhesive tape was equally successful with either tongue or wedge grafts.   A roll of ½-inch-wide adhesive tape was mounted on a roller at the side of the operator.   The graft was turned in the hand and the tape was applied in a spiral wrap over every part of the union and overlapped the edge slightly.   Only enough tape was used to make a water-tight covering over every part of the cut surface.   The tape was so applied that no more than two thicknesses of material circled the graft at any one point.   After a little practice it was possible to wrap 400 to 600 grafts in an hour.   The amount of tape used varied with the size of the grafts, but on an average approximately 110 yards of tape one half inch wide wrapped 1,000 grafts.

The tests with wrappers covered a wide range of conditions.   Results are reported for the years from 1925 to 1931, inclusive.   The unions of the first-year trees taken at random were examined by removing the soil to a suitable depth for inspection.   Sometimes only 50 tape-wrapped and 50 string-wrapped trees were examined, but at other times there were 100, 250, or 300 in each lot.

The results from the use of tape wrappers as compared with those from the use of commercial wrappers have been summarized for each trial (table 5) and have been grouped according to the age of the tree at the time the data were taken.   As explained earlier, no comparison should be made between the data on trees of different ages, for the trials are not comparable.

Table 5 presents the results of 145 trials with adhesive tape wrappers with a corresponding set of controls.   The trials with tape involved 55,326 trees, and the controls involved 55,105.   This table shows no significant difference in stand because of the different wrappers.   It shows variability in the amount of graft knot according to age of tree, variety, and the State in which the trees were grown.   It also indicates a distinct increase in the percentage of smooth trees correlated with the use of adhesive tape.   At the same time it emphasizes the variation between individual trials and indicates that different factors operate in the same nursery in different seasons and in different nurseries in the same season.

A summary of table 5 is given in table 6.

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[5] Manufactured under the name "nurseryman's tape."

TABLE 5.—*Effect of string [a] and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*

FIRST-YEAR TREES

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot [b]	Large knot [c]	Total examined	Smooth	Small knot [b]	Large knot [c]
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Iowa	Oldenburg	50	68	16	16	50	100	0	0
	do	50	56	34	10	50	92	8	0
	Wealthy	100	55	14	31	100	95	3	2
	do	100	84	10	6	100	100	0	0
	do	100	69	18	13	100	99	1	0
	do	100	84	2	14	100	97	2	1
	Yellow Transparent	50	84	8	8	50	100	0	0
	do	50	82	12	6	50	96	2	2
Kansas	Wealthy	50	54	26	20	50	96	2	2
	do	50	34	30	36	50	86	10	4
	do	50	36	16	48	50	86	10	4
	Yellow Transparent	300	71	29	0	300	78	22	0
	do	300	68	32	0	300	71	29	0
Minnesota	Bayfield	50	48	20	32	50	84	6	10
	do	50	58	16	26	50	82	10	8
	Okabena	50	36	26	38	50	84	6	10
	do	50	16	30	54	50	100	0	0
	Wealthy	50	40	20	40	50	90	4	6
	do	50	40	20	40	50	92	4	4
	do	50	46	30	24	50	86	12	2
	do	50	46	30	24	50	100	0	0
	do	50	42	18	40	50	100	0	0
	do	50	42	18	40	50	98	2	0
	do	50	52	14	34	50	94	6	0
Nebraska	do	100	83	3	14	100	99	0	1
	do	100	70	13	17	100	100	0	0
	do	100	70	13	17	100	96	3	1
	do	100	98	1	1	100	98	2	0
	do	100	92	4	4	100	100	0	0
Oklahoma	do	250	7	24	69	250	29	27	44
Wisconsin	do	250	53	27	20	250	94	2	4

SECOND-YEAR TREES

State	Variety	Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
Iowa	Ben Davis	155	72	1	27	215	94	0	6
	Delicious	50	94	2	4	50	100	0	0
	do	50	82	6	12	50	100	0	0
	do	50	84	4	12	50	96	2	2
	do	50	94	4	2	50	96	2	2
	do	50	96	4	0	20	100	0	0
	do	50	86	0	14	20	100	0	0
	do	50	98	0	2	50	96	0	4
	do	50	94	2	4	50	96	0	4
	Oldenburg	992	74	3	23	1,000	98	0	2
	do	399	79	2	19	1,014	94	0	6
	Dudley	50	88	8	4	50	88	0	12
	do	50	94	2	4	50	94	2	4
	Early Harvest	1,005	84	1	15	940	98	0	2
	Fameuse	568	79	2	19	1,009	97	0	3
	Florence (crab)	414	97	0	3	445	99	0	1
	Gano	573	80	0	20	695	97	0	3
	Golden Winesap	768	78	1	21	555	99	0	1
	McIntosh	1,012	84	2	14	1,020	94	1	5
	Red Siberian (crab)	512	96	0	4	575	98	0	2
	Wealthy	935	74	5	21	1,016	99	0	1
	do	50	70	16	14	50	96	2	2
	do	50	78	2	20	50	94	4	2
	do	1,000	93	0	7	1,000	97	0	3
	do	1,000	93	0	7	1,000	98	0	2
	do	1,000	90	0	10	1,000	97	0	3
	do	1,000	90	0	10	500	99	0	1
	do	279	86	0	14	1,330	90	0	10
	Whitney (crab)	1,034	91	0	9	1,049	99	0	1
	Yellow Transparent	259	88	2	10	286	96	1	3
	do	1,180	89	2	9	1,093	97	0	3

[a] Including string, waxed string, and waxed raffia.
[b] This class includes all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.
[c] This class includes all enlargements not classified as small.

TABLE 5.—*Effect of string and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*—Continued

SECOND-YEAR TREES—Continued

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Kansas	Ben Davis	654	49	4	47	649	60	5	35
	Jonathan	612	74	1	25	579	88	0	12
	....do	612	74	1	25	595	91	1	8
	....do	588	70	1	29	612	87	1	12
	McIntosh	567	89	1	10	431	95	0	5
	....do	559	89	1	10	518	95	0	5
	....do	1,005	58	3	39	512	76	2	22
	....do	534	56	2	42	515	73	1	26
	....do	550	56	4	40	551	80	1	19
	....do	112	61	3	36	534	83	0	17
	Wealthy	898	37	7	56	840	77	4	19
	....do	771	39	4	57	743	67	4	29
	....do	775	35	6	59	743	67	4	29
	....do	456	52	3	45	462	71	0	29
	Yellow Transparent	496	45	5	50	502	52	2	46
	Okabena	50	4	4	92	50	58	10	32
Minnesota	Wealthy	50	48	8	44	50	94	0	6
	....do	250	62	14	24	250	96	2	2
	Ben Davis	305	79	1	20	273	92	0	8
Missouri	Rome Beauty (dark red sport)	1,000	68	14	18	1,000	89	9	2
	....do	1,000	68	14	18	800	78	20	2
	Oldenburg	1,496	85	1	14	731	95	0	5
	Early Harvest	707	93	1	6	137	98	0	2
	Fameuse	1,080	89	1	10	996	97	0	3
	Maiden Blush	648	90	1	9	836	99	0	1
	Wealthy	178	57	2	41	216	80	2	18
	....do	243	57	2	41	316	82	3	15
	....do	243	57	2	41	342	84	3	13
	....do	500	47	1	52	45	87	0	13
	....do	500	47	1	52	68	88	2	10
	....do	1,000	94	0	6	1,000	98	0	2
	....do	1,000	81	1	18	1,000	89	1	10
	....do	1,000	46	2	52	1,000	94	1	5
	....do	250	74	3	23	250	90	1	9
	Winesap	227	90	1	9	320	97	0	3
	Yellow Transparent	693	47	2	51	583	87	0	13
Nebraska	Wealthy	50	82	6	12	50	92	6	2
	....do	50	82	6	12	50	100	0	0
	....do	250	66	21	13	250	89	4	7
	Delicious	447	84	0	16	238	98	0	2
	....do	438	72	1	27	310	87	2	11
Oklahoma	....do	473	94	1	5	465	97	0	3
	Jonathan	436	94	0	6	636	98	0	2
	York Imperial	367	66	1	33	441	89	1	10
Wisconsin	Wealthy	250	72	12	16	250	90	8	2
	....do	250	69	22	9	250	95	3	2
	....do	250	86	8	6	250	96	2	2

THIRD-YEAR TREES

State	Variety	Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
Iowa	Dudley	174	90	0	10	34	91	0	9
	....do	36	94	0	6	92	90	0	10
	Wealthy	101	80	0	20	104	94	0	6
	....do	270	85	1	14	182	96	0	4
Minnesota	....do	50	64	0	36	50	94	0	6
	....do	134	66	5	29	120	85	5	10
	....do	306	65	11	24	215	91	0	9
	....do	600	78	2	20	600	88	1	11
	....do	600	78	2	20	600	88	0	12
	....do	600	81	0	19	600	93	0	7
	....do	600	81	0	19	600	94	0	6
Nebraska	....do	600	80	0	20	600	80	0	20
	....do	600	80	0	20	600	89	0	11
	....do	376	81	0	19	375	81	0	19
	....do	361	82	1	17	382	94	0	6
	Whitney (crab)	533	85	1	14	529	93	0	7
	....do	645	86	1	13	742	95	1	4
	Yellow Transparent	961	79	1	20	972	87	1	12
	....do	813	84	0	16	765	87	0	13

TABLE 5.—*Effect of string and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*—Continued

THIRD-YEAR TREES—Continued

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Wisconsin	Bayfield	589	50	3	47	509	67	1	32
	Oldenburg	717	46	1	53	413	65	2	33
	Goodhue	495	60	0	40	542	68	0	32
	Northwestern Greening	402	71	5	24	736	71	2	27
	Perkins	763	66	0	34	310	74	1	25
	Red Wing	634	33	0	67	240	63	0	37
	do	471	66	5	29	482	82	4	14
	Wealthy	300	88	4	8	200	96	2	2
	do	1,243	48	0	52	424	73	0	27

FOURTH-YEAR TREES

State	Variety	Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
Minnesota	Bayfield	161	18	9	73	117	49	6	45
	do	184	29	8	63	265	36	7	57
	Okabena	111	8	1	91	57	42	2	56
	do	60	13	4	83	87	44	3	53
	Wealthy	308	34	5	61	362	67	2	31
	do	308	34	5	61	308	67	4	29
	do	272	14	2	84	331	59	3	38
	do	272	14	2	84	260	59	6	35

TABLE 6.—*Summary of table 5 on experiments showing total or average effect of string[a] and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted nursery apple trees of various ages*

Trials (number)	Wrapper	Age of trees	Total trees examined	Stand	Trees showing indicated condition at the union		
					Smooth	Small knot[b]	Large knot[c]
		Years	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
31	String	1	2,900	57	57	19	24
31	Tape	1	2,900	59	91	6	3
78	String	2	39,555	60	75	3	22
78	Tape	2	38,521	63	91	1	8
28	String	3	13,974	63	73	2	25
28	Tape	3	12,118	59	85	1	14
8	String	4	1,676	--------	21	4	75
8	Tape	4	1,787	--------	53	4	43
Summary:							
145	String	1–4	58,105	59	68	6	26
145	Tape	1–4	55,326	60	88	2	10

[a] Including string, waxed string, and raffia and wax.
[b] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.
[c] This class included all enlargements larger in cross measurements than half the diameter of the tree.

The superiority of adhesive-tape wrapping for reducing the percentage of enlargements on piece-root-grafted apple trees in most cases is beyond question. In none of the 145 trials in which there was more than 10 percent of disease were the string wrappers found to yield a higher percentage of smooth trees than the tape wrappers.

This alone places the mathematical probability that the tape is valuable in very significant figures.

The degree of superiority of the tape is difficult to estimate because of the variability of the results. In 20 trials there was less than 10 percent of disease development on the checks. Consequently, in such trials, control measures had little chance to show their value. On the other hand, some extreme differences occurred, such as differences of 40, 42, 48, and 56 percent in favor of the adhesive tape. The detailed results show differences ranging from 0 to 58 percent. The significance of the mean difference in relation to the standard deviation of the difference when at least 10 percent of knots were present was calculated, according to the method of Fisher (*5*), for trees of each age. When the value of P was less than 0.05, the mean difference in relation to the standard deviation of the difference was considered significant. The values of P were as follows: For the first-year trees, 0.02; for the second-year trees, 0.09; for the third-year trees, 0.12; and for the fourth-year trees, 0.02. In considering these values the variable factors already mentioned should be held in mind, for they make questionable the application of such mathematical treatment to some large portions of this data. It is obvious that certain influences which might affect the older trees, as explained earlier, did not affect the first-year trees. All the results with the fourth-year trees were taken in the same northern nursery during the same year. Consequently, the different trials were subject to fewer variables; this doubtless accounts for the low value of P. With a sufficiently large number of repetitions in several places and in successive years, the writers think that greater variations would probably be found. If similarly isolated groups of trials among the second- and third-year trees are taken, correspondingly low values of P are found.

Satisfactory control with adhesive tape was secured in the great majority of cases but not always. Examples may be found in table 5 in which the percentage of smooth trees from tape-wrapped grafts, while better than the controls, was only 29, 36, 42, 44, 49, 52, 58, 59, 60, etc. It appears from studies described by Riker and Hildebrand (*29*) and by Siegler and Piper (*41*) that results like these may be explained in two ways. (1) Infection of the union at the time of grafting may take place before the application of the adhesive-tape wrapper. This is perhaps pertinent when the discrepancy appears as it did once in Oklahoma on first-year trees. Such results resemble those secured from inoculations made at grafting time. (2) Infection may occur through injuries made by cultivation or by soil insects after the wrapper has decayed. This possibility has been already considered in relation to second-, third-, and fourth-year trees. This was doubtless the manner of infection on the first-year Yellow Transparent trees grown in Kansas, as recorded in table 5. Fortunately, detailed seasonal development records are available on these trees (*29*). Except for the one case in Oklahoma, these trees represent the worst failures on 1-year-old trees that the writers have experienced with tape wrappers.

The importance of infection at grafting time has been given consideration. Considerable emphasis has recently been placed on this one factor by Siegler and Piper (*41*). In an effort to discover the effect of adhesive tape upon infected unions, a number of grafts were inoculated with the hairy-root organism and then some were wrapped

with string and the others with tape. At the end of the first season, both the string-wrapped and the tape-wrapped lots showed 100 percent of hairy root. Repetitions of these trials the next year gave, respectively, 95 and 100 percent hairy root. These experiments showed that ordinary adhesive tape has little if any effect on bacteria present in the union at the time of grafting. In view of this evidence it appears that in many cases reported in tables 2 and 5 infection at the union at grafting time had comparatively little direct influence on the results. This is shown by the high percentage of smooth tape-wrapped trees at the end of the first season, suggesting that control measures based only on the importance of infection at grafting time may lead to disappointment in many cases. However, when considered from the standpoint of providing a source of inoculum for spread in the nursery by soil fauna during the latter part of the first season and during later growing seasons, as indicated by Riker and Hildebrand (*29*), it assumes more importance.

Difficulty with the adhesive tape has been encountered in three ways, none of which was serious. (1) The mechanical operation of wrapping the grafts and of cutting or tearing the tape at the proper place caused difficulty only at first. (2) Girdling of the trees during the first summer was found in about 10 percent of one planting of grafts. In this case too much tape had been wrapped about the unions, and the grafts were planted in very sandy soil. Dry weather prevailed for some time during the early part of the growing season, with the result that the tape wrapper was not sufficiently moist to decay. Even under these conditions no girdling was found on the trees that had received the right amount of wrapper. (3) A slight roughening of the bark beneath the plaster mass was noted in a few cases with certain varieties. Necrotic areas were observed which penetrated into the cortex for a short distance. Roughness of the union has sometimes been confused with the discolored residual particles of the plaster mass.

DISCUSSION

In the control of graft knots the seed used for growing seedling apple trees has been found to be of considerable importance. Since different varieties of apple trees have been shown to differ widely in their susceptibility to hairy root, it seemed probable that a similar difference in susceptibility might be found in seedlings grown from the seed of these varieties. A limited amount of evidence presented in this paper shows promise in this line of investigation. But aside from their relative resistance, which may be influenced not only by genetic constitution but also by conditions attending growth and harvesting, the seedlings may supply one or more of the other important factors.

The seedlings used for grafting may carry pathogenic bacteria. How these bacteria arrive at the surface of the seedlings is not yet perfectly understood. They may be in the soil where the seedlings are grown. However, a seemingly more important factor appears after the seedlings are dug. Ordinarily they are collected in bundles, placed in a heap, covered with packing (frequently old packing that might be classed as refuse), watered, and allowed to stand in order to "sweat off" the leaves. During this procedure it is obvious

that knot-producing bacteria on a very small percentage of infected seedlings or in the packing material might spread widely over the seedlings. There appear to be two possible remedies for this situation: (1) To prevent the spread of the bacteria; and (2) to destroy the bacteria by chemical treatments.

The bacteria carried on the surface of seedlings are aided in their entry into the union by certain cultural practices. In their effort to prevent desiccation of the seedling roots in the grafting room some nurserymen keep the roots wrapped in moist material until the minute of making the cut. Under such circumstances it has been observed repeatedly that the soil from the surface of the root may be carried over the cut surface by the knife, not only introducing any bacteria present into the union but also placing a layer of soil particles between parts of the scion and root and thus making union more difficult. Consequently, seedling roots that are clean and dry seem preferable during grafting to those that are wet and covered with soil. This appears to be quite important when the seedlings carry hairy-root bacteria, and deserves further experimental study.

The use of antiseptics on seedlings that carry hairy-root bacteria appears desirable. The determination of whether or not the infectious bacteria are carried on the surface of seedling roots is comparatively easy. By means of the technic developed by Patel (21) and Riker et al. (27), any well-equipped bacteriological laboratory might make the determinations. However, by no means all the apple seedlings carry infectious bacteria in sufficient numbers to be of primary consequence. Here again there is variation in different seedling nurseries during the same year and in the same nursery in different years. While some of the trials reported in tables 2 and 5 show that the bacteria were carried on the seedling and entered the union at grafting time, a larger number show that the union was invaded later on.

Wedge grafts appear to have no advantage over tongue grafts in the amount of graft knot developing. There was practically no difference when they were wrapped with tape. When the grafts were wrapped with string or raffia the wedge grafts had a somewhat greater tendency than tongue grafts either to come apart before planting or to send up sprouts from the root. On an average, the tongue grafts showed a slight advantage in stand.

The use of the adhesive-tape wrapper has been perhaps the most important single factor in the prevention of graft knots. Its function is rather complex. In the first place, inoculations at the union when the grafts were made indicated that the tape had little if any effect upon the entrance of bacteria at that time or on the development of infection by the bacteria that gained entrance. However, it had several other functions: (1) It prevented the further entrance of soil, water, and bacteria; (2) it reduced greatly any chance of injury to the union during various manipulations; (3) it encouraged better union between scion and root; (4) it prevented superficial development of excess callus; (5) some nurserymen have reported that it reduced the growth of mold at the union; and (6) it prevented root-chewing soil fauna from reaching the union for a number of months.

Root-chewing soil insects, including white grubs (Phyllophaga), wireworms (Elateridae), and fungus gnats (Mycetophilidae) (29),

may play an important role in the development of infectious hairy root not only during the first season but also during succeeding seasons. Obviously, control measures applied only at the time the grafts were made could have little effect on this factor except as they might reduce the amount of readily available inoculum. According to common entomological observation, the comparatively mild winters during the last few years probably enabled a greater number of insects to survive and have correspondingly increased their importance as a factor in the graft-knot problem. While abundant moisture in the soil favored the growth of the trees it also favored the production of callus, the activity of the insects near the surface, and the chances of entrance into the plants of any bacteria present in the soil. This serves in part to explain why graft knots are so much worse in moist than in dry years. In view of this situation, which deserves further study, such land as pasture that has been favorable to root-chewing insects seems relatively unsuited for apple grafts.

The following practices promise to contribute to the reduction of graft knots at the unions of apple trees grown from piece-root grafts:

(1) Use of apple seed from relatively resistant trees as soon as it is available.
(2) Treatment with an antiseptic of seedling roots suspected of carrying knot-producing bacteria.
(3) Use of clean dry roots when grafting.
(4) Use of a suitable wrapper, such as the adhesive tape described.
(5) Planting in soil which has been so handled that it is relatively free from root-chewing insects such as white grubs, wireworms, and fungus gnats.

SUMMARY

Graft knots of the types discussed in this paper cause severe losses in apple nursery stock propagated by piece-root grafting.

Nursery trees having infectious hairy root made, on an average, slightly less growth than smooth trees. Trees remained alive and grew slightly when all the roots but those in the hairy-root overgrowth had been removed.

The problem of controlling the overgrowths is complex, because there are several different kinds of enlargements, arising from different causes. The different kinds of overgrowths occur in various sorts and degrees of mixture.

The various overgrowths appear well distributed in the nursery. In the case of infectious hairy root, a certain amount of spread in the nursery row from hairy-root trees has been indicated.

A large percentage of the overgrowths occur at the unions. This percentage has been reduced on an average in the last 5 years, doubtless owing to improved nursery practice.

Examinations of a number of plantings have shown correlations between the length of time the trees stayed in the nursery row and the percentage of overgrowths. Although in some cases the initiation of most of the overgrowths could be traced to grafting time, in many others it was traced to the second or later seasons.

In some instances the surfaces of seedlings were found to carry hairy-root bacteria which were sources of inoculum at grafting time. In other instances this factor seemed of relatively little importance. The growing of seedling apple trees from the seed of relatively resist-

ant apple varieties gives promise of being a factor of some importance in the control of graft knots.

Antiseptic treatments of seedlings carrying bacteria that cause overgrowths seem to promise some measure of control.

Well-matched tongue grafts produced as many smooth unions as wedge grafts. Tongue grafts appeared to have certain minor advantages over wedge grafts.

Adhesive-tape wrapping appeared to be better than any other wrapping employed and to be the most important single factor among control measures. However, tape wrappers did not prevent infection at the time the grafts were made.

Control measures are discussed in relation to one another and to common nursery practice.

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TECHNIC FOR OBTAINING SPERMATOZOA FOR PHYSIOLOGICAL DAIRY STUDIES AND ARTIFICIAL INSEMINATION [1]

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INTRODUCTION

Semen may be collected from the vagina of the recently bred cow with the hand, by aspiration, or with a sponge. Semen from the vagina is satisfactory for determining whether the bull has ejaculated normal active spermatozoa during the mating, but it is unsatisfactory for use in physiological studies of spermatozoa because it is mixed with the secretions of the cow. Furthermore, collecting semen from the vagina for artificial breeding is wasteful.

A method of obtaining semen from the bull by massage of the accessory genital organs has been developed in the Bureau of Dairy Industry's physiological laboratory at Beltsville, Md. It has not been determined, however, what effect the continuous practice of obtaining semen in this way would have on the health and usefulness of the bull.

REVIEW OF THE LITERATURE

Komarov and Nagaev [2] designed a special rubber bag which they placed in the vagina of the cow. By careful technic in conducting the mating they were successful in obtaining a superior quality of semen as compared to that collected directly from the vagina with a sponge. Later, according to a report by Walton,[3] these workers used an artificial vagina and a "dummy" animal, which they claimed worked satisfactorily.

Case [4] in 1925 reported that "we procure the semen either by pressing on the seminal vesicles through the rectum, or from the vagina of the recently bred cow." In a letter to the authors in November 1932 Case described his method of massaging the seminal vesicles to obtain semen and also stated that he had used the method successfully 10 years ago in collecting semen for artificial impregnation.

ANATOMY OF THE BULL'S ACCESSORY GENITAL ORGANS

At Beltsville it was found that the seminal vesicles of the bull do not contain spermatozoa, but that the spermatozoa are in the ampullae of the ductus deferens.

The seminal vesicles and ampullae are easily identified and are so located that it is possible to manipulate the one without disturbing the other. It is more difficult to locate and manipulate the prostate of the bull because it consists of two parts and is protected by heavy muscle. The body of the prostate consists of a band which stretches across the neck of the urinary bladder and the origin of the urethra.

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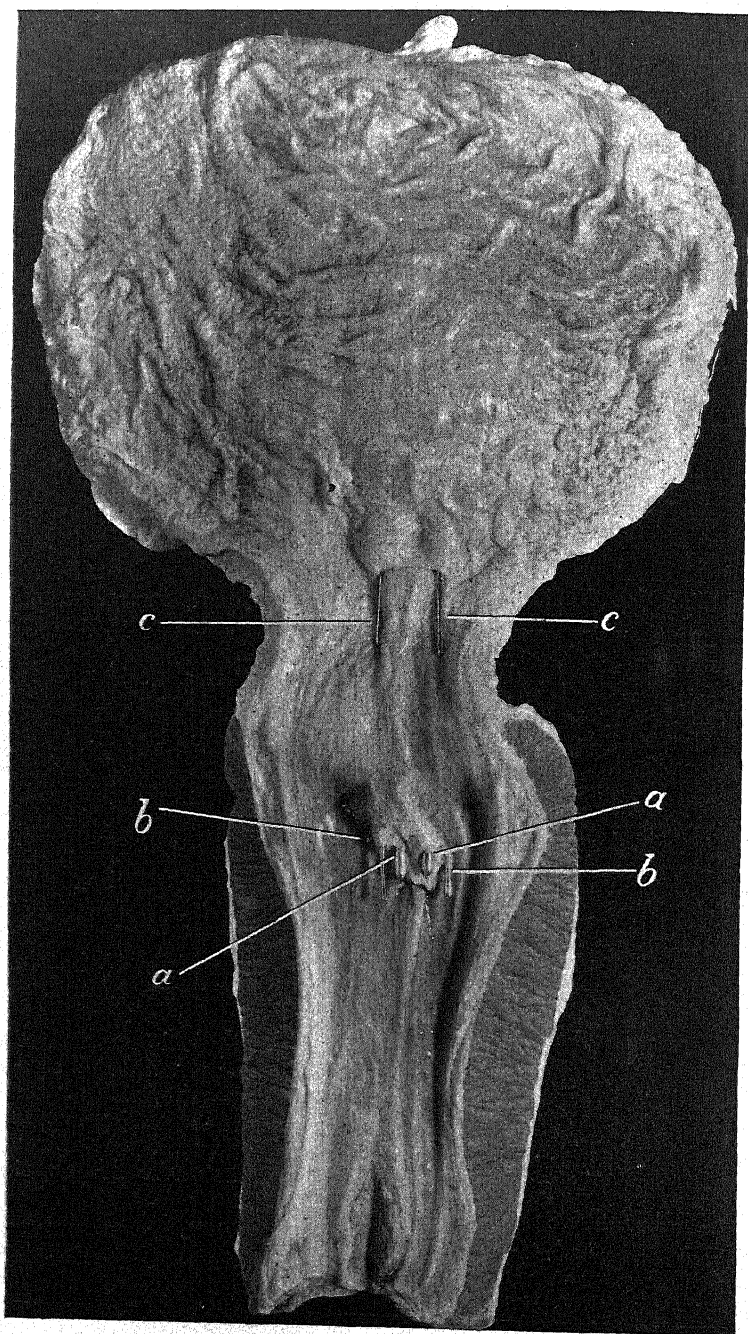
FIGURE 1.—Pelvic urethra and urinary bladder slit ventrally and laid open: *a*, Openings of ductus deferens; *b*, openings of seminal vesicles; *c*, uretral orifices.

It is about an inch and a half transversely and about half an inch in width and thickness. The pars disseminata surrounds the pelvic part of the urethra and is concealed by the urethral muscle (figs. 2 and 3).

As shown in figure 1 the ducts from the seminal vesicles and ductus deferens do not enter the urethra in a common opening; the ampullae of the ductus deferens have large lumen and the seminal vesicles have small tubules. The relation of these organs to each other is shown in figure 2.

METHOD AND RESULTS OF ITS USE

With a hand in the bull's rectum, from 7 to 10 inches, the seminal vesicles were massaged with backward strokes, and a turbid fluid flowed from the prepuce. It contained only epithelial cells in the majority of cases. In like manner the ampullae of the ductus deferens were massaged and a turbid fluid flowed out which contained only spermatozoa in the majority of cases. In some instances the ampullae were massaged first, but better results were obtained when the seminal vesicles were massaged first, because, while the ampullae were being massaged, the seminal vesicles released some of their fluid and both spermatozoa and epithelial cells were obtained. When the seminal vesicles were massaged first the epithelial cells came out, leaving only spermatozoa in the fluid obtained from the ampullae. Usually about 2 minutes of massaging gave excellent results.

In figure 3 the genital organs are shown replaced in the right half of a bull carcass. The weight of the urinary bladder has pulled the genital organs forward about an inch from the normal position in the live bull. The hand is shown massaging the seminal vesicles in figure 3, *A*, and the ampullae in figure 3, *B*.

Figure 4 shows the method of collecting the fluids as they flow out and figure 5 the comparative density of the fluids from the seminal vesicles and from the ampullae. Microscopical views of cells found in the two fluids are shown in figure 6.

Eighteen bulls ranging from $2\frac{1}{2}$ to 12 years in age were used in the first 100 trials to obtain semen by massage. Epithelial cells or debris were found in 100 samples of fluid from the seminal vesicles; and in 6 of these spermatozoa were found, always from bulls that had not been used for long periods. In these six cases it is probable that the ampullae were disturbed while the seminal vesicles were being massaged.

Of the 100 trials in massaging the ampullae 81 were successful and spermatozoa were obtained from 15 bulls. Thirty-one trials were made on one bull and each time enormous quantities of spermatozoa were obtained. No spermatozoa were obtained from the ampullae of three bulls. Two of these were tried only once and the other one twice. They were disposed of before further trials were made. Failure to obtain spermatozoa was experienced in 19 trials. It was assumed that the ampullae had been emptied just previous to the time of massaging. This was indicated by the volume and tone of the ampullae, the small flaccid tube yielding no spermatozoa and the large firm tube yielding many spermatozoa.

The quantity of fluid collected from the seminal vesicles at one time varied from 0.5 to 21 cc and that from the ampullae varied from 0.5 to 23 cc.

CONCLUSIONS

Massaging the accessory genital organs of the bull is a practical way of obtaining semen for physiological studies. For artificial breed-
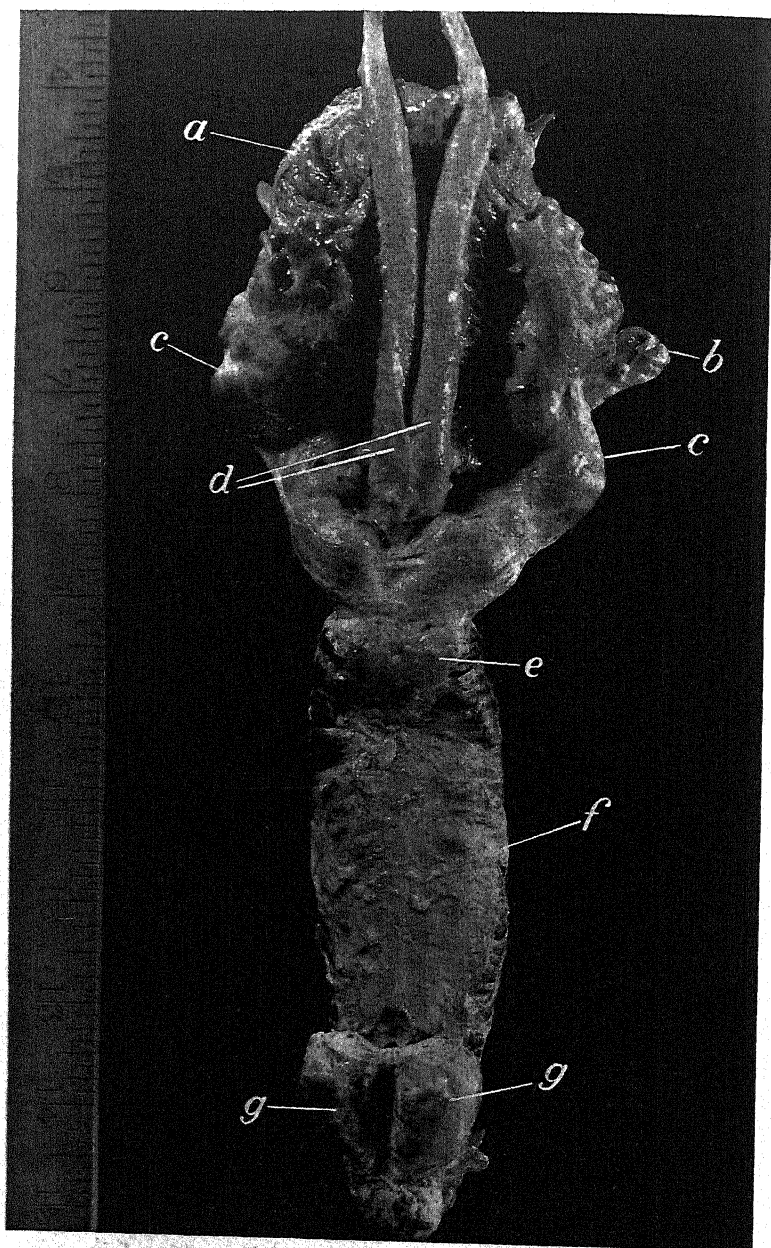
FIGURE 2.—Dorsal view of the internal organs of a bull: *a*, Bladder; *b*, ureter; *c*, seminal vesicles; *d*, ampullae; *e*, body of prostate; *f*, pelvic urethra; *g*, bulbo-urethral (Cowper's) glands.
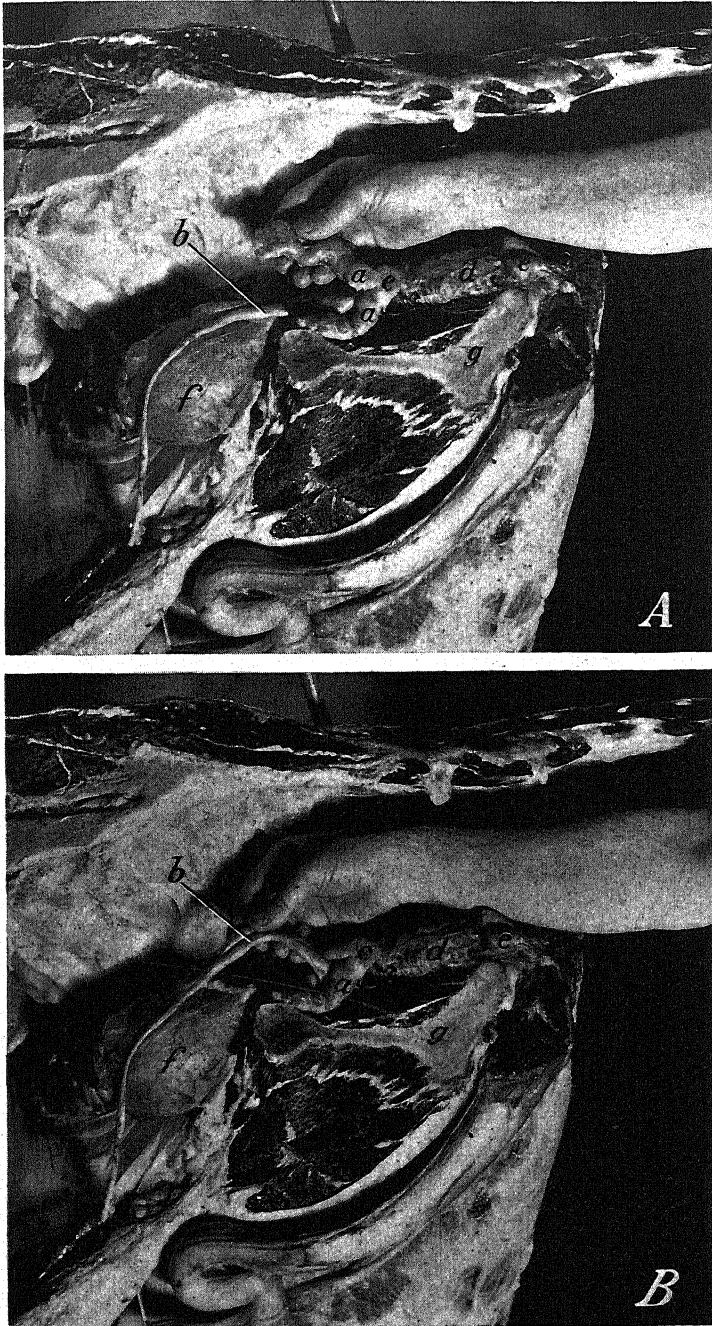
FIGURE 3.—Position of the genital organs of the bull and method of manipulating them: *A*, Massaging the seminal vesicles; *B*, massaging the ampullae of the ductus deferens. The organs are: *a*, Seminal vesicles; *b*, ampullae; *c*, body of prostate; *d*, pelvic urethra; *e*, bulbo-urethral (Cowper's) glands; *f*, urinary bladder; *g*, pubis

FIGURE 4.—Method of collecting semen with funnel and test tube.
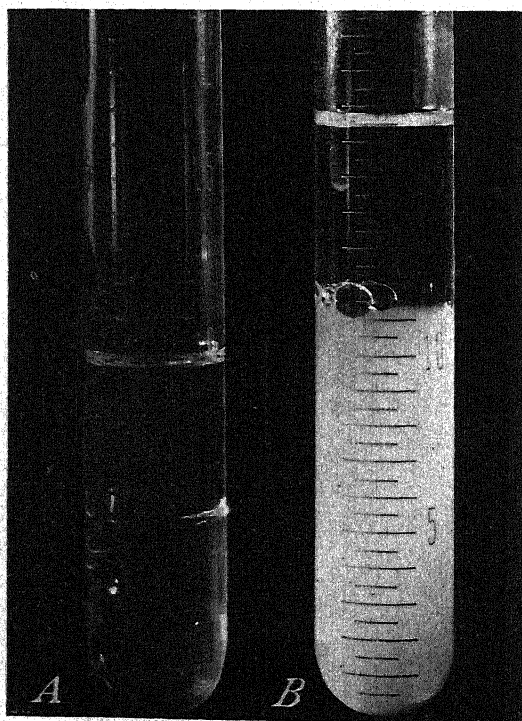
FIGURE 5.—Material collected from (*A*) seminal vesicles and (*B*) ampullae.

ing purposes it is desirable to massage the ampullae, for from this organ will be obtained the greatest volume of semen containing active spermatozoa. The method is also useful with valuable breeding bulls
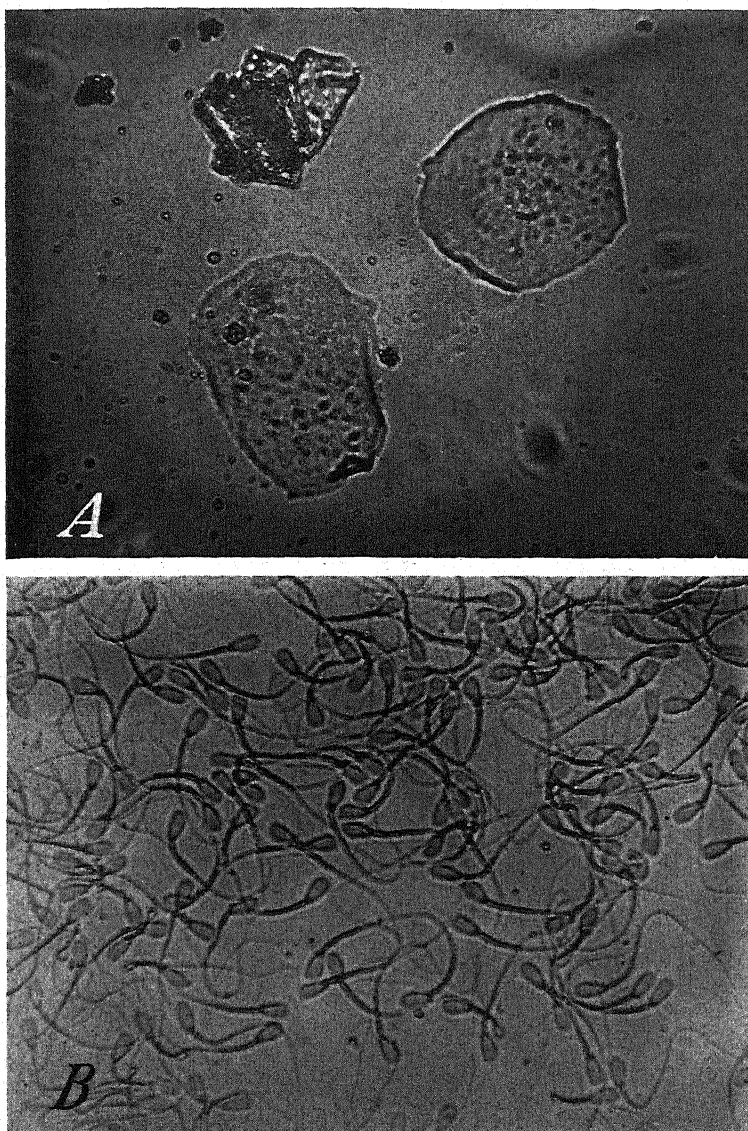
FIGURE 6.—*A*, Epithelial cells from seminal vesicles; *B*, spermatozoa from ampullae. × 450.

that are unable to serve cows in the normal manner because of injury. Other advantages of collecting semen directly from the bull for use in artificial breeding are that it prevents waste of semen and produces semen free from extraneous matter.
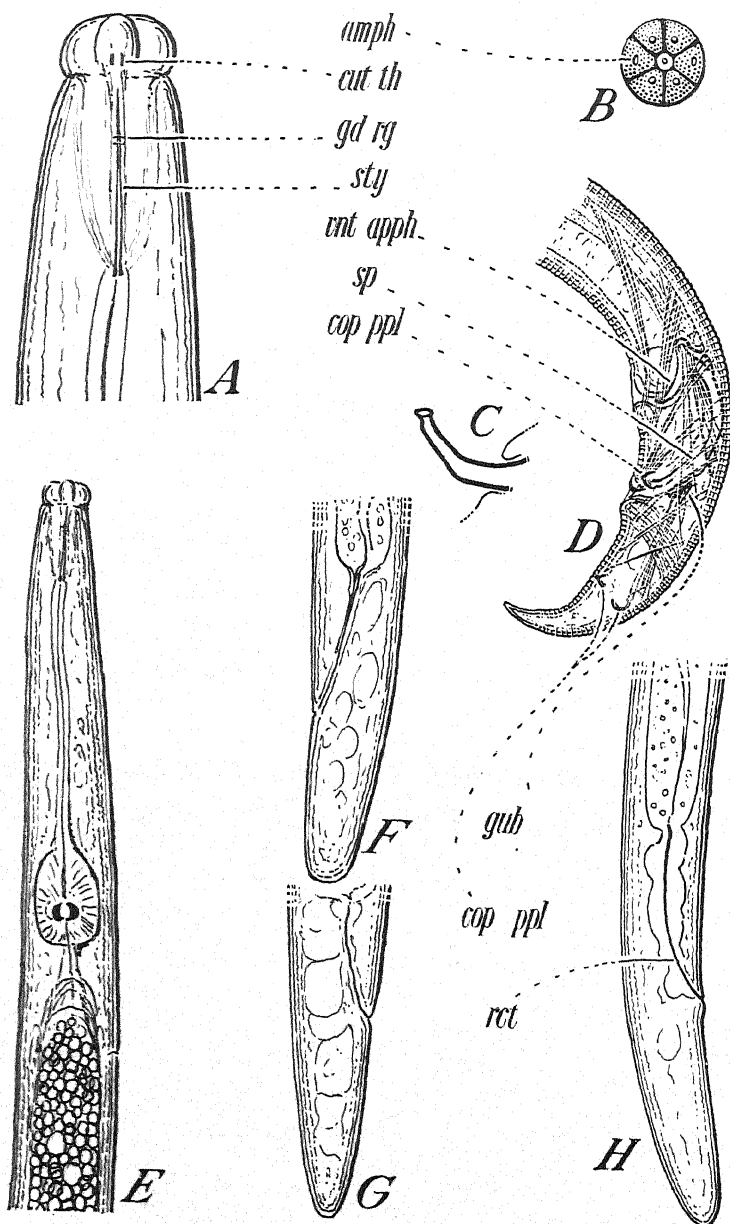
FIGURE 1.—*Aphelenchoides xylophilus*, n.sp. *A.*—Head of female: *cut th*, Cuticular thickening in cephalic portion of alimentary tract; *gd rg*, guiding rings of stylet; *sty*, stylet. × 1,370. *B.*—Front view of head: *amph*, Amphid. × 1,370. *C.*—Extruded spicula showing circular expansion. × 2,800. *D.*—Tail of male: *vnt apph*, Ventral apophysis; *sp*, spicula; *cop ppl*, copulatory papillae (three pairs); *gub*, gubernaculum. × 1,060. *E.*—Anterior end of larva. × 1,060. *F* and *G.*—Tails of larvae, showing variation in shape. × 1,060. *H.*—Tail of female: *rct*, Rectum. × 1,060.

to the "Fungi Imperfecti." However, according to the same author-
ity, this particular "isolation" was taken only a short distance (1
inch or less) from wood which was blue-stained by *Ceratostomella pini*
Münch, a fungus associated with *D. frontalis*. It is thought that the
nematode might also have been present in this blue-stained wood.

This constant association suggests that the nematode described
herein uses the insects as carriers and probably feeds on the various
fungi involved in the same association. Similar carrier relationships
between nematodes and insects are known, especially in connection
with bark beetles, dung beetles, flies frequenting fermenting substances,
etc. In this respect the observations made herein offer nothing new,
but the apparent specialization of this nematode to a life in wood and
its association with fungi of the blue-stain type merit special attention.

TECHNICAL DESCRIPTION

Aphelenchoides xylophilus, n.sp.

Like the other members of the genus, *Aphelenchoides xylophilus* is of slender
shape and has the following dimensions:

$$\female \; \frac{1.8 \quad 7.9 \quad 8.7 \quad 74. \quad 96.2}{1.0 \quad 1.5 \quad 1.5 \quad 1.6 \quad 1.0} \quad 0.9 \text{ mm.}$$

$$\male \; \frac{2.2 \quad 9.3 \quad 10. \quad M \quad 95.9}{1.2 \quad 1.6 \quad 1.6 \quad 2.0 \quad 1.5} \quad 0.77 \text{ mm.}$$

The cuticle is very finely annulated (8 annules to 6μ in the head region); the
head well set off; the tail of the larva and female more or less obtuse and slightly
longer than the rectum (fig. 1, *F, G, H*), that of the male conically pointed,
ventrally curved, and slightly longer than the spiculum (fig. 1, *D*). A front view
of the head shows a six-radiate cuticular structure. The lobes between the radii
carry the sense organs in the order typical for the genus, i.e., on the lateral lobe
the amphids, on each submedial lobe one papilla (fig. 1, *B*). The stylet is very
fine and about one and one-half times as long as the head is wide; its knobs are
minute; two fine guiding rings are present and the wall of the cephalic portion of
the alimentary tract is reinforced by short cuticular thickenings (fig. 1, *A*). It
seems that the esophageal glands open in the middle bulb of the esophagus in the
manner typical of the genus; the bodies of the glands, however, have a dorsal
situation outside the alimentary tract at the beginning of the intestine; they
extend to about 85μ behind the esophageal bulb and have a strictly serial arrange-
ment. The length of the rectum is about twice the anal body diameter. An
obscure excretory pore opens ventrad of the nerve ring (fig. 1, *E*). The vulva is
a narrow transverse slit but stands out rather well because the body narrows
suddenly behind it. The testis is outstretched forward to the right of the in-
testine, and ends about 300μ behind the esophageal bulb. The spicula resemble
those of other members of the genus but have in addition an extremely well-
developed ventral apophysis at the proximal end. In some specimens this
apophysis seemed to connect with the ventral body wall (fig. 1, *D*), but in others
no such connection was seen. Figure 1, *C*, shows the distal end of the spiculum
as forming a circular expansion. A small gubernaculum is present. The copula-
tory musculature is shown in figure 1, *D*. There are two pairs of large, somewhat
mammillate ventrosubmedial copulatory papillae (fig. 1, *D*), one pair at about
the middle of the tail and the other just in front of the anus. A third pair seems
to have a dorsolaterad position also in the middle of the tail.

DIAGNOSIS.—*Aphelenchoides* with an obtusely rounded conical tail in the larva
and in the female, but pointed in the male, with a fine, barely knobbed buccal
stylet. The spiculum of the male proximally with long ventral apophysis; a
short, lineate gubernaculum present; male tail with large mammillate copulatory
papillae; a pair ventrosubmedial in front of anus, a second pair ventrosubmedial
in the middle of the tail, and a third dorsolaterad also in the middle of the tail.
Associated with blue-stain and similar wood fungi.

TYPE HOST.—*Pinus palustris.*

RELATION OF BARBERRY TO THE ORIGIN AND PERSISTENCE OF PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS[1]

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INTRODUCTION

It is now well known (*4, 5, 6, 9, 13*)[2] that new physiologic forms of *Puccinia graminis* Pers. may arise as the result of hybridization between existing forms or varieties on barberry. The evidence at present available indicates that hybridization probably accounts principally for the origin of forms, although Stakman, Levine, and Cotter (*9*) have shown that mutation in parasitism may also occur, though probably rarely. There is little information, however, regarding the origin of new forms through hybridization on barberries in nature. For this reason the writers made studies during the past several years to ascertain whether new physiologic forms could be obtained from aecial material and from uredial material near barberries. Preliminary statements of the results have been published (*7, 12*). These and other data have been combined with the results of earlier surveys and are presented in the following pages. Data are given also on the results of "selfing" pycnia resulting from inoculations with telial collections of unknown identity.

The writers were particularly interested in the varieties of *Puccinia graminis* that attack the common small grains; hence inoculations were made on barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.), or on wheat (*Triticum vulgare* Vill.),[3] oats and rye (*Secale cereale* L.), to obtain preliminary indications of the identity of the variety of rust in question. Barley is susceptible to both the *tritici* and the *secalis* varieties; hence, it sometimes was used in the preliminary inoculations to "screen out" the *poae* and *agrostidis* varieties, neither of which develops well on it. The determination of physiologic forms within rust varieties was made by revised keys originally developed by Stakman and Levine (*8*) for physiologic forms of *P. graminis tritici* Eriks. and Henn.; by Bailey (*1*) for physiologic forms of *P. graminis avenae* Eriks. and Henn., and by Cotter and Levine (*3*) for physiologic forms of *P. graminis secalis* Eriks. and Henn.

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The relatively low percentage of infection resulting from inoculating with aeciospores may be due to several causes. *Puccinia graminis poae* Eriks. and Henn. and *P. graminis agrostidis* Eriks. and Henn.[4] are very prevalent on barberries in certain regions, and many of the collections probably were of these varieties. Then, too, most of the material was sent through the mails and much of it was not in good condition when received. Further, the germination of aeciospores is somewhat more uncertain and considerably more capricious than that of urediospores; and aeciospores do not usually retain their viability more than 3 weeks, even when kept cool and fairly dry.

VARIETIES ISOLATED

VARIETIES FROM AECIAL COLLECTIONS

Of 675 aecial collections of *Puccinia graminis* tested during the past 13 years, only 281 caused infection on wheat, oats, rye, or barley (table 1). It seems likely, therefore, that many of the collections were of the *poae* or the *agrostidis* variety. Of the 281 collections that caused infection on the common small grains, 96, or 34.2 percent, were of the *tritici* variety; 179, or 63.7 percent, were of the *secalis* variety; and only 6, or 2.1 percent, were of the *avenae* variety.

TABLE 1.—*Isolation of Puccinia graminis varieties from rusted barberries in the field, 1920–32*

Year	Collections tested	Total cultures identified	Isolations of *Puccinia graminis* var.—					
			Tritici		Secalis		Avenae	
	Number	Number	Number	Percent	Number	Percent	Number	Percent
1920	8	4	3	75.0	1	25.0		
1921	14	6	5	83.3	1	16.7		
1922	9	3	1	33.3	2	66.7		
1923	6	2			2	100.0		
1924	12	6	1	16.7	5	83.3		
1925	9	5	3	60.0	2	40.0		
1926	19	12			11	91.7	1	8.3
1927	27	19	8	42.1	9	47.4	2	10.5
1928	93	60	23	38.3	35	58.4	2	3.3
1929	64	41	18	43.9	23	56.1		
1930	131	42	16	38.1	26	61.9		
1931	124	32	12	37.5	19	59.4	1	3.1
1932	159	49	6	12.2	43	87.8		
Total	675	281	96	34.2	179	63.7	6	2.1

It seems quite likely that the high percentage of collections of *Puccinia graminis secalis* is due to the fact (1) that quackgrass (*Agropyron repens* (L.) Beauv.) is susceptible to this variety, (2) that this grass is very prevalent in the Northern States, where barberries become rusted, and (3) that many of the remaining barberry bushes are along fence rows, in pastures, on the edges of wood lots, along streams, and in other uncultivated places, where quackgrass is likely to be abundant. Furthermore, *Hordeum jubatum* L. and other species of *Hordeum* are very susceptible to *P. graminis secalis*, as are also various species of *Elymus*. There is therefore opportunity for *P. graminis secalis* to develop abundantly. *P. graminis tritici* attacks not only wheat but also the same grasses that *P. graminis secalis* attacks, with the exception of *Agropyron repens*. As just explained, however, the great

[4] The varietal name as written by Eriksson and Henning is *agrostis*. In order to conform with the other varietal names, however, the writers prefer to use the genitive form, *agrostidis*, as originally used by Eriksson.

abundance and the advantageous habitat of the last-named grass give
P. graminis secalis the advantage, despite the fact that the acreage of
wheat attacked by *P. graminis tritici* is far greater than the acreage of
rye attacked by *P. graminis secalis*.   The relative paucity of collec-
tions of *P. graminis avenae* probably is due to the fact that *Dactylis
glomerata* L., *Festuca* spp., *Alopecurus* spp., *Glyceria*, and the other
grasses susceptible to this variety usually are not particularly
abundant near barberry bushes (*11*).

VARIETIES FROM UREDIAL MATERIAL NEAR INFECTED BARBERRIES

In addition to determining the relative prevalence of the varieties
of *Puccinia graminis* occurring in the aecial stage on barberry bushes in
nature, the writers made a similar study of uredial material on grains
and grasses in the immediate vicinity (within 100 yards) of infected
bushes.   This study includes only such rust as almost certainly
resulted from infected bushes.   The results are summarized in table 2.

TABLE 2.—*Isolations of Puccinia graminis varieties from uredial collections obtained
in close proximity to infected barberries, 1919–32*

Year	Cultures identified	Isolations of *Puccinia graminis* var.—					
		Tritici		Secalis		Arenae	
	Number	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
1919	3	2	66.7	1	33.3		
1920	3	1	33.3	2	66.7		
1921	4	2	50.0	2	50.0		
1922	9	3	33.3	6	66.7		
1923	3			1	33.3	2	66.7
1924	4			3	75.0	1	25.0
1925	5	2	40.0	3	60.0		
1926	7	2	28.6	4	57.1	1	14.3
1927	21	9	42.9	8	38.1	4	19.0
1928	27	16	59.3	5	18.5	6	22.2
1929	7	4	57.1	3	42.9		
1930	8	5	62.5	1	12.5	2	25.0
1931	19	12	63.2	2	10.5	5	26.3
1932	18	14	77.8	4	22.2		
Total	138	72	52.2	45	32.6	21	15.2

A total of 138 collections of *Puccinia graminis* were identified.   Of
these, 72, or 52.2 percent, were of the *tritici* variety; 45, or 32.6 per-
cent, of the *secalis* variety; and 21, or 15.2 percent, of the *avenae*
variety.   The fact that the percentage of collections of the *tritici* va-
riety was higher than that of the *secalis* variety, whereas the opposite
was true of the aecial collections, is probably due largely to conscious
selection.   The writers were most interested in determining physiologic
forms of the *tritici* variety and therefore made special effort to collect
grasses and grains likely to be infected with it.   Hence the percentages
do not necessarily give as accurate an indication of the relative preva-
lence of the varieties in nature as those for the aecial collections.
It is clear that a high percentage of *Puccinia graminis* on barberries
in the Northern States is of the *secalis* variety.   This variety of rust
probably is more closely dependent on barberry for persistence from
season to season than are the *tritici* and *avenae* varieties.   This is
partly because very little rye is grown in the South, where the uredial
stage of *Puccinia graminis* can survive the winter.   It is evident that

there is appreciable development of stem rust of rye only in those regions where there are barberries. Stem rust of rye can be prevented, for practical purposes, by eradicating barberries. While it is perfectly clear that barberry eradication reduces the severity of rust attacks on wheat and oats also, investigations by the writers have shown that the uredial stage of these rusts often survives the winter in the far South and, under favorable conditions, urediospores may be blown northward, to cause infection on wheat and oats in the Northern States.

PHYSIOLOGIC FORMS ISOLATED

Attempt was made to identify the physiologic forms, both in aecial and uredial collections. In addition, barberries were inoculated in the greenhouse with collections of teliospores and the pycnia were "selfed", as described later. The primary object was to find out to what extent new physiologic forms were arising through hybridization in nature. It was desired also to ascertain to what extent barberries make possible the persistence of existing forms. Theoretically barberries should be of great importance in these respects, and, practically, the results show that they are.

PUCCINIA GRAMINIS TRITICI

PHYSIOLOGIC FORMS FROM AECIAL COLLECTIONS

The identity of the physiologic forms represented in the 94 isolations of *Puccinia graminis tritici* was determined, 26 forms being
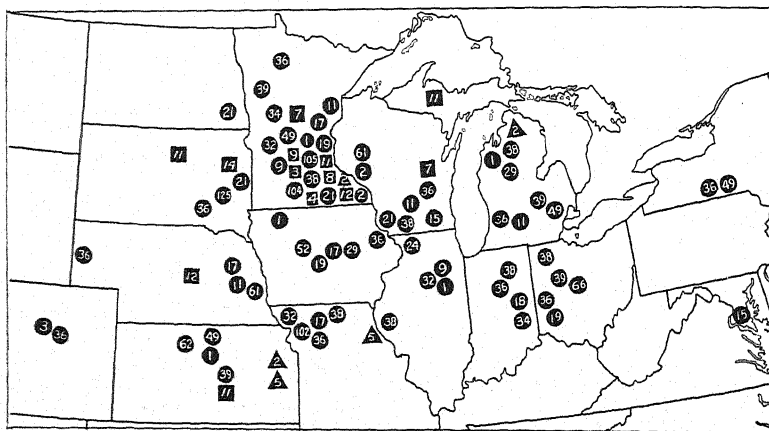
FIGURE 1.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*, isolated from naturally infected barberries. Circles indicate variety *tritici;* squares, *secalis;* triangles, *arenae.*

identified. Some of them were of frequent occurrence and wide distribution, others rare and limited; some were quite virulent, others fairly innocuous (table 3 and fig. 1; see also table 5).

It is worthy of note that so limited a number of isolations should have yielded so many different physiologic forms. This becomes especially striking when the number of physiologic forms isolated from field collections of infected grain plants is considered. In the latter case, 82 physiologic forms have been identified from more than 8,000

cultures made in the course of physiologic-form surveys; that is, on an average, not more than one form from each 100 uredial collections; whereas from aecial collections a different form was procured, on an average, from every fourth culture, although this ratio probably would not have persisted had more aecial collections been identified.

TABLE 3.—*Physiologic forms of Puccinia graminis tritici isolated from aecial collections, by States, 1920–32*

Form	Number of times form was found in—															
	Colorado	Illinois	Indiana	Iowa	Kansas	Maryland	Michigan	Minnesota	Missouri	Nebraska	New York	North Dakota	Ohio	South Dakota	Wisconsin	Total
1		1		1	1		1	2								6
2								1							1	2
3	1															1
9		1						1								2
11							1	1		1					1	4
15						1									1	2
17				1				1	1	1						4
18			1													1
19				1				1					1			3
21								2				1		1	1	5
24	1															1
29				1			1									2
32		1						1	1							3
31			1													2
36	1		1	2			1	8	2	2	1		1	2	3	24
38		1	2				1	2	2				1		2	11
39					2		1	1					1			5
49					1		1	3				1				6
52				1												1
61											1				1	2
62						1										1
66														1		1
102										1						1
104								1								1
105								2								2
125														1		1
Total	2	5	5	7	5	1	7	28	7	5	2	1	5	4	10	94
Percent	2.1	5.3	5.3	7.5	5.3	1.1	7.5	29.8	7.5	5.3	2.1	1.1	5.3	4.2	10.6	100
Number of forms	2	5	4	6	4	1	7	15	5	4	2	1	5	3	7	26

Form 36 was most common and most widely distributed. It was isolated 24 times in a total of 94 identifications, and it was found in 11 of 15 States in which collections were made. Next in order of occurrence and distribution was form 38, with a total of 11 isolations from 7 States. This was followed by a considerable drop in both occurrence and distribution was form 38, with a total of 11 isolations from 7 States. This was followed by a considerable drop in both occurrence and distribution, with 6 isolations each of forms 1 and 49 from 5 and 4 States, respectively. Forms 3, 18, 24, 52, 62, 66, 102, 104, and 125 were isolated only once each from the aecial material collected.

Forms 62, 102, 104, and 105 never have been isolated from any source other than rusted barberry. The original collections of forms 61 and 66 were made from naturally infected barberries, although subsequently they were obtained also from rusted grain in the field.

These facts indicate strongly that in addition to spreading much inoculum early in the season barberry bushes are extremely important in at least two respects: (1) They enable many physiologic forms to persist from season to season and (2) they make possible the origin of

new forms through hybridization. In 1919 it was pointed out by Stakman, Levine, and Leach (*10*) that the number of physiologic forms of *Puccinia graminis* seemed to be greater in those regions of the United States where there were many barberry bushes than in those where there were few. The results presented in this paper support this opinion and indicate clearly that barberry bushes should be eradicated in order to reduce the number of physiologic forms as well as to reduce the amount of inoculum, especially that of the early spring.

Of the forms isolated only from barberries and therefore probably produced on them during recent years, form 62 is the most virulent. It may cause heavy infection on all the differential hosts except Vernal and Khapli emmers. It is one of the most virulent of all forms, differing from form 15 only in its inability to infect Vernal normally
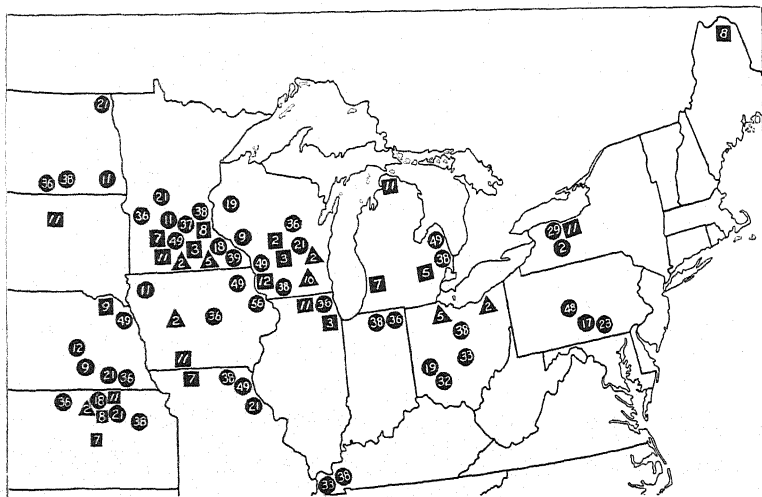
FIGURE 2.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*, collected in the vicinity of infected barberries. Circles indicate variety *tritici;* squares, *secalis;* triangles, *avenae.*

and in its tendency to produce type x infection on Marquis, Reliance, and Kota wheats; on these wheats form 15 produces type 4 infection (table 5). Under favorable conditions type x may develop sufficiently to cause heavy rust attack; hence form 62 must be considered potentially dangerous. The other forms isolated only from barberries are not particularly virulent, although it is worthy of note that on Vernal emmer both form 104 and 105 produce type x infection, which is a heavier infection than that caused by most other forms.

PHYSIOLOGIC FORMS FROM UREDIAL MATERIAL NEAR INFECTED BARBERRIES

The 71 collections of *Puccinia graminis tritici*, made on grains and grasses near infected barberries and completely identified, comprised 19 physiologic forms, a different form for approximately every four collections (table 4, fig. 2). These results support those obtained from inoculations with aeciospores. Here again it is clear that barberries are responsible for the persistence of many physiologic forms. When inoculations were made with urediospores collected at random,

in regions where barberries rust and in those where they do not, a different form appeared in 1 collection in 100, on an average. But when inoculations were made with urediospores collected near rusted barberries, a different form was obtained in 1 culture of every 4.

TABLE 4.—*Physiologic forms of Puccinia graminis tritici isolated from uredial collections obtained near infected barberries, by States, 1919–32*

| Form | Number of times form was found in— | | | | | | | | | | | | | | |
	Illinois	Indiana	Iowa	Kansas	Michigan	Minnesota	Missouri	Nebraska	New York	North Dakota	Ohio	Pennsylvania	Virginia	Wisconsin	Total
2										1					1
9								1						1	2
11			1			1					1				3
12							1								1
17												1			1
18				2		2									4
19											1			1	2
21				1		3	1	1			1			2	9
23												1			1
29										1					1
32											1				1
33											1		1		2
36	1	1	1	2		4		1			4			2	16
37						1									1
38		2		1	1	1	2				1	1	1	4	14
39						1									1
48												1			1
49			2			1	2	1	1					2	9
56			1												1
Total	1	3	5	6	2	15	4	5	2	7	4	3	2	12	71
Percent	1.4	4.2	7.1	8.5	2.8	21.1	5.6	7.1	2.8	9.9	5.6	4.2	2.8	16.9	100
Number of forms	1	2	4	4	2	8	3	5	2	4	4	3	2	6	19

Forms 36 and 38 were the most prevalent, constituting 22.54 percent and 19.72 percent, respectively, of the total collections identified. Form 38 has been very prevalent in the soft red winter wheat area of the United States, where there still are many barberries; it is also very abundant in northern Mexico. Forms 21 and 49 were next in order of prevalence. By consulting table 5 it will be seen that these four forms could cause heavy rust on the principal types of wheat commonly grown in the wheat-growing regions of the northern half of the United States. Marquis and similar varieties are susceptible to three of these forms; the durums are resistant to forms 36 and 49, susceptible to form 21, and under favorable environmental conditions they are susceptible to form 38, which causes a type x+ infection. Reliance is immune from forms 21 and 49 but is susceptible to forms 36 and 38 (table 5). This variety reacts like Kanred, which often is immune in the field. It rusts heavily, however, when forms like 36 and 38 are present. Kota, which reacts like the widely grown variety, Ceres, is at least moderately susceptible to all four forms but seldom becomes heavily rusted in the field because of morphologic resistance.

Form 48 has never been found in the United States except near rusted barberries, although it has been reported several times from Canada, where it was first collected in 1929, on wheat. It is, however, of rare occurrence.

PUCCINIA GRAMINIS SECALIS

The physiologic forms of 27 aecial collections of *Puccinia graminis secalis* were identified, 9 forms being found. It seems significant that so many forms were isolated from so few collections. On an average, a different form was isolated from every third collection. Forms 7 and 11 were the most prevalent, constituting 25.93 and 29.63 percent, respectively, of the total (tables 6 and 8, and figs. 1 and 2).

TABLE 5.—*Types of infection [a] on differential varieties of wheat caused by physiologic forms of Puccinia graminis tritici isolated from aecia and from uredia near infected barberries, 1919–32*

Form	Reaction of differential varieties											
	Jen-kin	Mar-quis	Re-liance	Kota	Arnaut-ka	Min-dum	Spel-mar	Ku-banka	Acme	Ein-korn	Ver-nal	Kha-pli
1	4	4	0	3	1	1	1	3	3	3	0	1
2	4	2	2	2	1	1	1	1	3	3	1	0
3	4	4	4	3	1	1	1	1	3	3	1	0
9	4	4	0	3	4	4	4	4	3	3	4	1
11	4	4	3	3	4	4	4	3	3	3	1	1
12	4	4	4	3	4	1	1	1	3	3	1	0
15	4	4	4	3	4	4	4	3	3	3	4	1
17	4	4	0	3	4	4	4	3	3	3	1	1
18	4	4	4	3	1	1	1	3	3	3	1	1
19	4	2	0	3	4	4	4	3	3	3	0	1
21	4	4	0	3	4	4	4	4	3	1	0	1
23	4	2	1	1	1	1	1	3	3	3	0	0
24	4	4	0	2	4	4	4	3	3	3	1	0
29	4	4	0	3	x	x	x	x	x	3	1	1
32	4	4	4	3	x	x	x	x	x	3	1	1
33	4	2	4	1	1	1	1	4	3	3	1	1
34	4	4	4	4	4	4	4	4	3	1	0	1
36	4	4	4	3	1	1	0	x	3	3	0	1
37	4	4	0	3	4	4	4	x	3	3	1	1
38	4	2	4	3	x	x	x	x	x	4	1	1
39	4	2	4	3	4	3	4	4	3	4	1	1
48	4	1	0	1	x	x	x	x	4	4	1	1
49	4	4	0	4	1	1	0	x	3	1	0	1
51	4	2	3	0	0	0	0	4	3	3	4	0
52	4	4	4	4	1	1	1	x	4	4	4	1
56	4	3	3	3	1	1	1	3	3	1	1	1
61 [b]	4	4	0	3	0	0	0	x	4	4	0	0
62 [c]	4	x	x	x	4	4	4	4	4	3	0	1
66 [b]	4	2	4	0	0	0	0	x	1	3	0	0
67 [d]	4	4	4	4	1	2	2	x	x	3	4	1
96 [d]	4	x	4	x	4	4	4	4	3	3	1	1
101	4	4	4	4	1	0	0	x	x	3	0	1
102 [c]	4	0	1	0	0	0	1	1	0	3	0	1
104 [c]	4	x	0	0	0	0	0	0	0	3	x	0
105 [c]	4	x	0	3	0	0	0	x	3	3	x	1
125	4	4	4	4	0	0	0	x	4	1	0	1
127 [d]	4	4	3	3	1	1	1	x	x	0	0	1

[a] 0 signifies immune; 1, very resistant; 2, moderately resistant; 3, moderately susceptible; 4, very susceptible; x, heterogeneous (uredia very variable, various types of infection appearing on the same leaf, not due to mechanical mixture.
[b] First isolated from barberries.
[c] Isolated only from barberries.
[d] From selfing only.

TABLE 6.—*Physiologic forms of Puccinia graminis secalis and P. graminis avenae, isolated from aecial collections, by States, 1920–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	South Dakota	Wisconsin	Total
P. graminis secalis:									
3					1				1
4					1				1
5		1							1
7					6			1	7
8					1				1
9					3				3
11	3		2		2		1		8
12					3	1			4
14							1		1
Total	3	1	2		17		1	2	1	27
P. graminis avenae:									
2	1			1	2				4
5	1					1			2
Total	2			1	2	1			6

NUMBER OF FORMS

	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	South Dakota	Wisconsin	Total
P. graminis secalis	1	1	1		7		1	2	1	9
P. graminis avenae	2			1	1	1			2

Form 5 is the only form that had not been isolated previously from uredial material. As it was subsequently obtained also from uredia near barberries, it probably had been formed recently.

From 28 uredial collections of *Puccinia graminis secalis* near infected barberries, 8 physiologic forms were isolated, again a large number of forms in proportion to the number of collections. Forms 7 and 11 were again the most prevalent (table 7).

PUCCINIA GRAMINIS AVENAE

Only six aecial collections of *Puccinia graminis avenae* were identified. Forms 2 and 5 occurred in the ratio of 2 to 1. Both are extremely common and widely distributed in the United States; consequently no particular significance attaches to their occurrence on barberries. However, an entirely new form, designated form 10, was obtained from oats near rusted barberries in Wisconsin. A special note on this form has been published by Cotter (*2*); hence details will not be repeated here, beyond calling attention to the fact that it may reasonably be concluded to have resulted from hybridization on barberries in nature. It is important to note that form 10 is much more virulent on the Richland group of oat varieties than either form 2 or form 5 (table 8).

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TABLE 7.—*Physiologic forms of Puccinia graminis secalis and P. graminis avenae isolated from uredial collections made near infected barberries, by States, 1919–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Illinois	Iowa	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	New York	Ohio	South Dakota	Wisconsin	Total
P. graminis secalis:													
2												1	1
3	2					1						1	4
5					1								1
7			3		1	1	3	1					8
8			2	1		1							4
9									1				1
11	1	1	1			2	1			1		1	8
12												1	1
Total	3	1	6	1	4	6	1	1	1		1	3	28
P. graminis avenae:													
2		1	1			1				1		1	5
5						2				1			3
10												2	2
Total		1	1			3				2		3	10

NUMBER OF FORMS

	Illinois	Iowa	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	New York	Ohio	South Dakota	Wisconsin	Total
P. graminis secalis	2	1	3	1	3	4	1	1	1		1	3	8
P. graminis avenae		1	1			2				2		2	3

TABLE 8.—*Types of infection produced on differential varieties of rye and oats caused by physiologic forms of Puccinia graminis secalis and P. graminis avenae, respectively, isolated from aecia and from uredia near infected barberries, 1920–32*

Form	Varieties of rye and relative susceptibility to *P. graminis secalis*					Varieties of oats and mean infection type		
	Rosen	Swedish	Prolific	Dakold	Colorless	Minrus	Richland	Joanette
2	88.1	15.8	40.0			2	1	4
3	88.5	83.4	81.4	60.9	88.3			
4	93.3	81.8	62.6	61.3	96.9			
5	84.3	6.9	85.7			2	1	x
7	89.3	62.7	60.4	38.0	88.8			
8	67.8	41.4	62.1	13.6	79.4			
9	87.3	52.4	81.2	19.6	86.7			
10						2	4	x
11	87.4	46.7	58.7	17.4	86.6			
12	86.1	63.5	81.2	36.7	87.8			
14	85.8	39.9	53.9	10.8	68.3			

PHYSIOLOGIC FORMS FROM AECIA RESULTING FROM SELFED PYCNIA

To obtain still further information on the role of barberries in producing new physiologic forms and in perpetuating old ones, a number of "selfings" were made. The method was to inoculate barberries with collections of telial material, then transfer pycnial nectar from certain pycnia to others of the same collections, and inoculate differential hosts with the progeny of spores resulting from inoculations with spores from single aecia. There is, of course, no way of knowing certainly whether forms isolated were already present in the telial

stage, whether they resulted from segregation, or whether new combinations were made. The essential fact brought out by the results, however, is that in proportion to the number of cultures isolated a large number of forms was always obtained. From some of the aecia resulting from inoculation with teliospores, 2 forms, and in one case, 4 forms, were obtained. The average ratio between the number of forms isolated and the number of telial collections used for inoculation was 3 to 2. The ratio was approximately the same with *Puccinia graminis tritici* and *P. graminis secalis*, being 1.62 to 1 and 1.56 to 1, respectively. As has been previously pointed out, when determinations of physiologic forms of *P. graminis tritici* were made from uredial material collected at random, one physiologic form was isolated from approximately every 100 collections. From aecial material resulting from inoculating barberries with teliospores in the greenhouse, however, the average number of forms isolated from each collection was 0.39. This would seem to indicate, again, that the
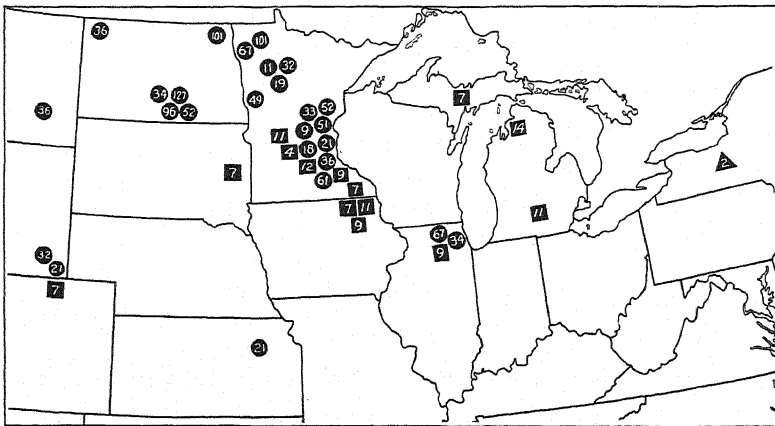
FIGURE 3.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*. segregated after passing through susceptible barberry. Circles indicate variety *tritici;* squares, *secalis;* triangle, *avenae.*

eradication of barberries will result in a diminution of the number of forms.

From a total of 30 cultures of *Puccinia graminis tritici*, 17 different physiologic forms were isolated (table 10). Those obtained most commonly were forms 67 and 101, not forms 36 and 38, which were most frequently isolated from aecial collections and from uredial collections made near barberries in nature. Nevertheless, form 36 follows forms 67 and 101 in order of frequency and is very similar to them in pathogenicity, but form 38 was not obtained at all. The complete list of forms isolated is given in table 10. (See also fig. 3.)

Forms 67, 96, and 127, which were isolated in these experiments, have never been obtained from uredial material. Of these, forms 67 and 96 had previously been produced as a result of artificial hybridization and form 127 was first isolated as a result of selfing but has been produced subsequently by hybridization also. Form 101 had been identified from uredial material collected in Bulgaria but has not been found in nature in North America. Its only recorded appearance on this continent is on barberries on which pycnia were selfed.

Forms 7, 9, 11, and 12 of *Puccinia graminis secalis* also were isolated from aecia produced on barberries in nature and from uredia obtained near barberry bushes. Of these, 7 and 11 have been most prevalent throughout. Forms 4 and 14 were obtained once each from the aecial material and from the products of selfing, but form 14 has never been obtained from uredial isolations.

It seems probable from the results given above that still other new forms might be produced if there were opportunity for certain telial material to cause infection on barberries in nature.

TABLE 9.—*Physiologic forms of Puccinia graminis varieties isolated from aecia produced as a result of selfing pycnia on barberries inoculated with collections of teliospores, 1926–32*

Year	Number of selfings [a] and number of forms isolated of *P. graminis*							
	Total		Var. *tritici*		Var. *secalis*		Var. *avenae*	
	Selfings	Forms isolated	Selfings	Forms isolated	Selfings	Forms isolated	Selfings	Forms isolated
1926	1	4	1	4				
1927	2	5	1	2	1	3		
1928	1	1	1	1				
1929	5	7	2	2	3	5		
1930	2	3	1	1	1	2		
1931	6	7	4	5	2	2		
1932	14	21	11	19	2	2	1	0
Total	31	48	21	34	9	14	1	0
Ratio of number of forms to selfings	1.55:1		1.62:1		1.56:1		0:1	

[a] This indicates the number of times barberries were inoculated with teliospore collections. Several pycnia were selfed in each case and inoculations made with aeciospores from the resulting aecia. Urediospores were then used for inoculating differential varieties.

DISCUSSION AND CONCLUSIONS

The results presented in the foregoing pages show clearly that barberries are important in two ways besides that of producing abundant inoculum early in the spring: (1) They enable new physiologic forms to arise through hybridization, and (2) they apparently enable many forms to persist and multiply.

The results of these field studies support the opinion, based on previous greenhouse experiments, that new forms arise on barberries in nature. Four forms of *Puccinia graminis tritici* have been isolated only from naturally infected barberries in the field, and two others were isolated from barberries before they were found elsewhere. Form 5 of *P. graminis secalis* has been isolated only from aecia on barberries and from uredia in close proximity to infected barberries, while form 10 of *P. graminis avenae* has been found only near infected barberries. The writers are convinced that these forms were of recent origin, indicating that new forms are still arising on susceptible barberries. The significance of the results is increased by the fact that these forms had not been found previously in extensive physiologic-form surveys made each year since 1917.

There is strong circumstantial evidence that the numerous physiologic forms now in existence have arisen principally as a result of hybridization. Despite very extensive experiments with physiologic

TABLE 10.—*Physiologic forms of Puccinia graminis varieties isolated from aecia produced as a result of selfing pycnia on barberries inoculated with collections of teliospores, by States, 1926–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Colorado	Illinois	Iowa	Kansas	Michigan	Minnesota	Montana	New York	North Dakota	South Dakota	Wyoming	Total
P. graminis tritici:												
9						1						1
11						1						1
18						1						1
19						1						1
21				1		1					1	3
32						1					1	2
33						1						1
34		1							1			2
36						1	1		1			3
49						1						1
51						1						1
52						1			1			2
61						1						1
67		1				3						4
96									1			1
101						3			1			4
127									1			1
Total		2		1		18	1		6		2	30
P. graminis secalis:												
4						1						1
7	1		1		2	4				1		9
9		1	1			1						3
11			1		1	12						14
12						1						1
14					1							1
Total	1	1	3		4	19				1		29
P. graminis avenae:												
2									1			1

NUMBER OF FORMS

	Colorado	Illinois	Iowa	Kansas	Michigan	Minnesota	Montana	New York	North Dakota	South Dakota	Wyoming	Total
P. graminis tritici		2		1		14	1		6		2	17
P. graminis secalis	1	1	3		3	5				1		6
P. graminis avenae									1			1

forms since 1916, the writers have observed only two definite cases of mutation in parasitism, which are believed to be the only ones on record for *Puccinia graminis*. Color mutations are not infrequent, but mutation in pathogenicity seems rare. On the other hand, there is abundant evidence that new forms arise frequently through hybridization. It seems reasonable, therefore, to assume that most physiologic forms have arisen in this way.

The large number of forms in regions where barberries become heavily rusted and the smaller number in regions where the aecial stage is rare also support the hypotheses just stated. As already pointed out by Waterhouse(*13*), the presence of a large number of forms in the Mississippi Valley of North America and of a small number in Australia seems significant. Furthermore, in the rather isolated "Inland Empire" of the Pacific Northwest of the United States, where barberries rarely become rusted, there seem to be relatively few forms of *Puccinia graminis tritici*, although wheat has long been grown extensively in the region and club wheats, which are sus-

ceptible to nearly all forms known in North America, have been grown commonly.

It is a striking fact, also, that so large a number of forms can be isolated from aecia and from uredia on grains and grasses in close proximity to barberry bushes. For example, a different form of *Puccinia graminis tritici* was isolated from approximately every third collection of aecial material, whereas a different form was isolated from only each 100 collections of uredial material collected at random (tables 11 and 12).

TABLE 11.—*Summary of the identity and number of physiologic forms of Puccinia graminis varieties isolated from aecial and telial collections, and uredial collections made near barberries, 1920–32*

Form	Number of times each form was found							
	Aecial collections from infected barberries			Uredial collections near infected barberries			Telial collections selfed on barberries	
	Var. tritici	Var. secalis	Var. arenae	Var. tritici	Var. secalis	Var. arenae	Var. tritici	Var. secalis
1	6							
2	2		4	1	1	5		
3	1	1			4			
4		1						1
5		1	2		1	3		
7		7			8			9
8		1			4			
9	2	3		2	1		1	3
10 [a]						2		
11	4	8		3	8		1	14
12		4		1	1			1
14		1						1
15	2							
17	4			1				
18	1			4			1	
19	3			2			1	
21	5			9			3	
23				1				
24	1							
29	2			1				
32	3			1			2	
33				2			1	
34	2						2	
36	24			16			3	
37				1				
38	11			14				
39	5			1				
48				1				
49	6			9			1	
51							1	
52	1						2	
56				1				
61 [b]	2						1	
62 [c]	1							
66 [b]	1							
67 [d]							4	
98 [d]							1	
101							4	
102 [c]	1							
104 [c]	1							
105 [c]	2							
125	1							
127 [d]							1	
Total	94	27	6	71	28	10	30	29
Number of forms	26	9	2	19	8	3	17	6

[a] Found near infected barberries only.
[b] First isolated from barberries.
[c] Isolated only from barberries.
[d] From selfing only.

TABLE 12.—*Physiologic forms isolated as compared with collections of Puccinia graminis varieties cultured*

| Variety | Physiologic forms isolated from collections of designated spore stages | | | | | | | |
| | Aecial | | Uredial | | Telial | | Weighted average [a] | |
	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]
P. graminis tritici_____	3.62:1	27.66	3.74:1	26.76	1.76:1	56.67	3.15:1	31.72
P. graminis secalis_____	3.00:1	33.33	3.50:1	28.57	4.83:1	20.68	3.65:1	27.38
P. graminis avenae_____	3.00:1	33.33	3.33:1	30.00	_____	_____	3.20:1	31.25
Weighted average for spore stages_____	3.43:1	29.13	3.63:1	27.52	2.57:1	38.98	3.28:1	30.51

[a] As a basis of comparison, the ratio for *P. graminis tritici* was approximately 100:1 in the general physiologic-form survey, when collections were made at random in the United States and Mexico, that is, from approximately each 100 collections a different form was obtained.
[b] Ratio of number of collections cultured to number of physiologic forms found.
[c] Percentage of physiologic forms in terms of the number of collections cultured.

Evidently barberries are responsible, therefore, not only for the production of new forms, but also to a considerable extent for their persistence; and in previous publications abundant evidence has been presented showing that barberries also are responsible for the dissemination of a tremendous amount of inoculum early in the growing season.

SUMMARY

The writers have investigated the role of barberries in the production and perpetuation of physiologic forms of *Puccinia graminis* in nature.

During the past 13 years inoculations were made on the common small grains with material from 675 aecial collections of *Puccinia graminis* obtained from the northern part of the United States. Of these, 281 caused infection, 34.2 percent being of the *tritici* variety, 63.7 percent of the *secalis* variety, and only 2.1 percent of the *avenae* variety.

The relative prevalence of the different varieties of *P. graminis* probably is governed to a considerable extent by the distribution of wild grasses susceptible to the different varieties.

The varietal identity of 138 uredial collections of *P. graminis* obtained within 100 yards of rusted barberries also was determined, with the following percentages: *tritici*, 52.2 percent; *secalis*, 32.6 percent; and *avenae*, 15.2 percent. These percentages probably were affected by a certain amount of conscious selection of hosts known to be susceptible to certain rust varieties.

The results given above, supplemented by other observations, indicate that stem rust of rye (*P. graminis secalis*) is almost wholly dependent on barberries for its persistence in the United States.

From 94 aecial collections of *P. graminis tritici*, 26 physiologic forms were isolated, a different form from approximately every 4 collections, whereas from about 8,000 uredial collections made at random over a period of years a different form was isolated from about every 100 collections.

Of the physiologic forms isolated from *P. graminis tritici*, forms 36 and 38 were the most prevalent.

Four forms (62, 102, 104, and 105) never have been isolated from any source other than rusted barberries. Forms 61 and 66 were isolated first from rusted barberries but subsequently were isolated from rusted wheat also.

From 71 uredial collections of *P. graminis tritici* made near rusted barberries, 19 physiologic forms were obtained, one of which (form 48) never has been found elsewhere in the United States, although reported several times from Canada.

The results indicate that barberries in nature are responsible for the production of new physiologic forms of *P. graminis tritici* as well as for the persistence of numerous forms.

Twenty-seven aecial collections of *P. graminis secalis* comprised 9 physiologic forms, and 28 uredial collections obtained near rusted bushes comprised 8 forms, a far larger number in proportion to the number of collections than in the case of uredial collections made at random. Forms 7 and 11 were the most prevalent; form 5 has been obtained only from aecia or uredia formed near infected barberries, and form 14 was isolated only from aecial collections or was the product of "selfing" on barberry.

Only six aecial collections of *P. graminis avenae* were identified, forms 2 and 5, which are widely distributed in the United States, being isolated. A new form (10), however, was isolated from oats near rusted barberries. It is far more virulent on the Richland group of oat varieties than either of the other two forms mentioned.

When barberries were inoculated with telial material in the greenhouse, the ratio between the number of forms isolated and the number of telial collections used for inoculating was 2 to 5. For example, 17 forms were isolated from 30 cultures of *P. graminis tritici* and 6 forms from 29 cultures of *P. graminis secalis*.

Forms 67, 96, and 127 of *P. graminis tritici*, isolated as a result of inoculating barberries with teliospores, never have been obtained from uredial material in the field, and another form (101) has never been found in the United States except on artificially inoculated barberries, although it was isolated from uredial material collected in Bulgaria.

Two physiologic forms (4 and 14) of *P. graminis secalis* were isolated from artificially inoculated barberries and from naturally infected bushes in the field, but form 14 has never been isolated from uredial material.

It is concluded that in nature barberries are important in the production and persistence of physiologic forms and in the persistence of certain varieties of rust, especially *P. graminis secalis* and probably *P. graminis agrostidis* and *P. graminis poae* also.

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SIZE AND ARRANGEMENT OF PLOTS FOR YIELD TESTS WITH CULTIVATED MUSHROOMS [1]

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INTRODUCTION

Numerous studies have been made to determine the most efficient plot technic for various agronomic and horticultural crops under different conditions. A bibliography on this subject has been published recently.[3]

In the few papers dealing with the results of yield tests in experiments with the cultivated mushroom (*Agaricus campestris* L.) a discussion of plot technic has been entirely neglected. Yet plot technic is of unusual importance in mushroom tests since there is normally more variability in mushroom beds than in ordinary field plots and the experimenter has an unusual opportunity to control conditions. Furthermore, the number and size of experimental plots must be reduced to a minimum because the labor and expense of yield tests is increased tremendously by the mushroom's habit of fruiting continuously for 3 or 4 months.

In commercial practice mushrooms are usually grown on shelf beds in windowless sheds or houses. An average mushroom house is about 60 feet long and contains 2 tiers of 5 or 6 shelf beds in which the individual beds are 24 inches apart. The beds are 5 or 6 feet wide and run the entire length of the house, except for a narrow passageway at each end of the house.[4] As a result of the placing of uprights and bed supports at 4-foot intervals along the bed (fig. 1), the beds are normally subdivided into small sections 4 feet wide and extending across the bed.

These structural features suggest at least four convenient units of bed space which might be used for yield trials—entire houses, tiers of beds, beds, and sections.

Practical experience soon shows that entire houses are not suitable, for differences in compost heaps and in the prevalence of fungus diseases and pests in the various houses more than counterbalance the advantages gained by the large area harvested. Tiers of beds are preferable to entire houses, since comparisons may be made among test areas located in the same house, and since the beds are made from the same compost heap. For most experiments, however, this arrangement is not satisfactory because it precludes replication of the experimental areas owing to the fact that there are only two tiers of beds in a house. These objections do not apply to the use of beds

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or smaller plots, such as sections or groups of sections, for test units. Therefore, for most experiments beds or parts of beds would seem to be, a priori, the proper experimental units.

The choice between entire beds and smaller plots depends largely on the comparative variability of the yields from these units. The experiments outlined in this paper were designed primarily to throw light on this question and on the problem of plot arrangement.

MATERIAL AND METHODS

The experiments were conducted as simple uniformity trials. They were made in the Department's experimental mushroom cellar at the Arlington Experiment Farm, Rosslyn, Va., and in commercial houses

FIGURE 1.—Experimental mushroom beds at Arlington Experiment Farm, Rosslyn, Va., divided into ½-section plots 2 feet wide and extending across the bed. Note the placing of upright supports at 4-foot intervals; this is the standard arrangement in commercial houses and the beds as a matter of convenience are usually filled with sections as unit areas, by unloading 10 or 12 bushels of compost at one time in the area between upright supports.

at Downingtown and Coatesville, Pa. The manure was composted in the regular manner, and the beds were filled with compost at the rate of approximately 1 bushel for 2 square feet of bed space. At Arlington Farm and Downingtown the beds were filled by emptying 10 bushel baskets into each 4-foot section on beds 5 feet wide and 12 bushel baskets into each section on beds 6 feet wide. At Coatesville 2 sections were filled at one time with 24 bushels of compost. In each experiment the composting in the heap was done as uniformly as possible, the same batch of spawn was used for the entire surface, and the casing soil was from a single source in each house. The sections at the ends of the beds were discarded because the yields from these sections are frequently reduced by excessive drafts. The picking was done at intervals of from 1 to 3 days until the beds were practically exhausted. In this connection it should be noted that the production period was terminated at Arlington Farm by a spell of hot weather

and the lower beds, which were slow to start, were injured more than the upper beds.

At Arlington Farm the total experimental area consisted of 5 beds each divided into 10 plots one half section (2 feet) wide (fig. 1). At Downingtown 5 beds were each divided into 10 plots one section (4 feet) wide. At Coatesville 4 beds were each divided into 10 plots one section (4 feet) wide. The yields given in table 1 for the separate plots are the sums of all the daily yields taken over a period of 3 months for each plot.

The "analysis of variance" method, devised and described by Fisher,[5] was used for analyzing the data. An arithmetical procedure was followed similar to that outlined by Fisher and Wishart.[6]

ANALYSIS OF YIELD DATA FROM UNIFORMITY TRIALS

ARLINGTON EXPERIMENT FARM, ROSSLYN, VA.

The yields of the 50 individual plots on the 5 experimental beds at Arlington Farm are given in table 1. If the 10 plots are considered as separate treatments and the beds as replicate blocks of these treatments, an analysis of variance, as shown in table 2, may be calculated that helps to clear up many of the points with which we are concerned.

TABLE 1.—*Yields of mushrooms in 3 uniformity trials*

YIELD PER 10-SQUARE-FOOT PLOT IN EXPERIMENTAL HOUSE AT ARLINGTON EXPERIMENT FARM, ROSSLYN, VA.

Bed no.	Yield from plot no.—									
	1	2	3	4	5	6	7	8	9	10
	Ounces	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*
1	382	263	242	312	184	226	283	273	272	262
2	286	338	239	336	239	297	216	231	220	266
3	153	154	143	175	167	203	209	170	203	201
4	145	143	158	170	176	174	127	167	133	153
5	162	148	158	194	176	181	183	239	210	193

YIELD PER 24-SQUARE-FOOT PLOT IN A COMMERCIAL MUSHROOM HOUSE AT DOWNINGTOWN, PA.

	Pounds	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*
1	21	21	25	24	28	30	20	24	25	24
2	36	27	32	27	28	27	24	38	30	23
3	20	20	20	29	15	24	36	29	21	30
4	28	25	16	22	23	24	20	21	20	27
5	24	21	19	36	26	33	28	24	32	23

YIELD PER 24-SQUARE-FOOT PLOT IN A COMMERCIAL MUSHROOM HOUSE AT COATESVILLE, PA.

1	31	36	42	37	50	45	42	40	43	37
2	42	41	41	42	41	31	45	47	45	42
3	43	40	41	40	40	50	37	45	46	39
4	31	34	42	35	42	37	41	41	41	43

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TABLE 2.—*Analysis of variance of yields of mushrooms harvested in 3 locations from plots of different sizes, and arrangement*

ARLINGTON EXPERIMENT FARM, ROSSLYN, VA. (YIELD IN OUNCES)

| Arrangement of plots corresponding to fig. 2 | Plots | | | Blocks or beds (total area) | Variation | Degrees of freedom [a] | Sum of squares [b] | Variance [c] | z [d] | Standard deviation [e] | Coefficient of variability of— | | Lowest significant difference (percent between means of 5 replications) [h] | Efficiency (per cent on an area basis) [i] |
	Width	Per block	Area of each (sections or parts of sections)								Single plot [f]	Mean of 5 replications [g]		
	Feet	*Number*		*Number*										
A	2	10	½	5	Between beds	4	115,731.6	28,932.90	1.5440	36.32	17.24	7.71	23.13	100
					Between columns	9	10,432.9	1,159.21						
					Interaction	36	47,486.0	1,319.06						
					Total	49	173,650.5							
B	4	5	1	5	Between beds	4	231,463.5	57,865.87	1.4316	57.47	13.6	6.08	18.21	80.3
					Within beds	20	66,058.5	3,302.92						
					Total	24	297,522.0							
C	6	3	1½	5	Between beds	4	322,002.0	85,504.4	1.3168	78.35	12.4	5.54	16.62	68.3
					Within beds	11	67,543.0	6,140						
					Total	15	389,545.0							
D	4	4	2 half	5	Between bed	4	165,479.3	41,369.82	1.6733	39.41	9.57	4.28	12.84	162.2
					Within beds	15	23,297.5	1,553.16						
					Total	19	188,776.8							

COMMERCIAL MUSHROOM HOUSE AT DOWNINGTOWN, PA. (YIELD IN POUNDS)

				Source	Degrees of freedom	Sum of squares[b]	Mean square[c]	z[d]	S.E.[e]	C.V.[f]	[g]	[h]	[i]
E	4	10	1	5	Between beds	4	261.6	65.40	0.47468	5.01	19.7	8.81	26.43	100
					Between columns	9	154.4	17.15						
					Interaction	36	906.0	25.17						
					Total	49	1,322.0							
F	8	5	2	5	Between beds	4	523.0	135.8	.47328	7.26	14.8	6.62	19.86	88
					Between columns	4	50.8	12.7						
					Interaction	16	864.0	52.7						
					Total	24	1,437.8							

COMMERCIAL MUSHROOM HOUSE AT COATESVILLE, PA. (YIELD IN POUNDS)

				Source	Degrees of freedom	Sum of squares[b]	Mean square[c]	z[d]	S.E.[e]	C.V.[f]	[g]	[h]	[i]
G	4	10	1	4	Between beds	3	74.2	24.9		4.3	10.6	4.74	14.22	100
					Between columns	9	214.4	23.8						
					Interaction	27	500.8	18.5						
					Total	39	789.4							
H	8	5	2	4	Between beds	3	134	44.5		5.9	7.2	3.22	9.66	108
					Between columns	4	281	70.2						
					Interaction	12	488	37.3						
					Total	19	903							
I	8	4	2	4	Between beds	3	100.7	33.90		4.23	4.1	1.8	5.5	331
					Between columns	3	126.5	42.16						
					Interaction	9	160.8	17.87						
					Total	15	388.0							

[a] Number of independent comparisons.

[b] Sum of the squares of the deviations from the mean yield.

[c] Variance or mean square = $\dfrac{\text{sum of squares}}{\text{degrees of freedom}}$.

[d] z = one half of the difference between the natural logarithms of the mean squares between beds and of error.

[e] Standard error (standard deviation) = square root of variance.

[f] Coefficient of variability = standard error in percentage of the mean yield = $\dfrac{\text{S.E.} \times 100}{\text{mean yield}}$.

[g] Coefficient of variability divided by the square root of 5.

[h] $\dfrac{3 \times C.V.}{\sqrt{5}}$. (Odds approximately 20 to 1 against the occurrence due to chance of a difference as great or greater.)

[i] See p. 976.

In the reduction of the data the first objective was to determine the comparative efficiency of beds taken as a whole and of smaller plots such as sections or groups of sections. The data indicate higher variability between beds than between smaller plots (within beds) and lend themselves to the application of Fisher's z test to determine the statistical significance of this difference. When Fisher's table VI is consulted it is apparent that the observed value of z exceeds the 1-percent point in all cases, and it may be concluded that the differences are significant. The odds are more than 100 to 1 that such a difference would not occur as a result of chance variation. Since the variance between beds was significantly greater than the variance within beds, the data indicate that greater precision can be expected with smaller plots, and justify the arrangement of plots in the beds so that the variance between beds can be eliminated in calculating the standard error. In practice this would mean the random distribution of treatments on the beds with the restriction that each treatment shall occur once on each bed.

Conceivably, there might be conditions in the mushroom house that would bring about a consistently larger yield at one end of several of the beds analogous to the fertility gradients frequently encountered in soil-heterogeneity studies. Under these conditions there probably would be justification for arranging the plots in 5 by 5 Latin squares, in which the plots in each bed would constitute the rows and the plots one above the other or opposite one another in the house would constitute the columns of the square. Since all the plots have been treated uniformly the justification for such a procedure can be tested from the data in table 1 by calculating and comparing the variance between columns with the variance within columns and blocks. This comparison also is given in the analysis of variance shown in table 2. In this case the variance due to the interaction of beds and columns (error) is larger than the variance between columns, indicating that there would be no significant gain in the precision of the experiment by arranging the plots in a Latin square.

The next point of interest to be considered is the gain or loss in efficiency from the use of different-sized plots, such as one-half section (10 square feet), full section (20 square feet), or 1½ sections (30 square feet). In a series of analysis of variance as shown in table 2 the standard error in percentage of the mean, or coefficient of variability, was found to be reduced from 17.24 for one-half sections to 13.6 for entire sections, and 12.4 for 1½ sections (triple plots). On a plot basis this indicates a gain in precision with increase in plot size, but full-section plots take up twice as much space as the ½-section plots and 1½-section plots take up three times as much space. This raises the question of the relative efficiency of ½-section plots, full-section plots, and triple plots when the same area is used in each case. Immer [7] gives a method of determining the efficiency of plots of varying size and shape, calculated on the basis of variance per unit area of land, that can be used to answer this question. With the small plot as a standard, the relative efficiency of the larger plots was found by multiplying the square of the coefficient of variability per plot by the quotient of the area of the larger plot divided by the area of the small plot and expressing the result in percentage of the

[7] IMMER, F. R. SIZE AND SHAPE OF PLOT IN RELATION TO FIELD EXPERIMENTS WITH SUGAR BEETS. Jour. Agr. Research 44: 649–668, illus. 1932.

square of the coefficient of variability of the small plot used as a standard. When calculated in this way, if the ½-section plot is considered as 100 percent efficient it becomes apparent that on an area basis the efficiency of the full section is only 80.3 percent and the efficiency of the 1½-section plot only 68.3 percent. In other words, under the conditions exemplified by the plots studied, 10 replications of ½-section plots are preferable to 5 replications of entire sections; and 15 replications of ½-section plots are preferable to 5 replications of 1½-section plots.

Theoretically it seems probable that the customary method of filling the beds with compost and of using sections in the beds as units for filling would cause a greater variability between normal sections than between areas of similar size that do not coincide with the sections on the bed. The data at hand offer a means of testing this hypothesis. It is merely necessary to shift over one plot before pairing the yields of the ½-section plots to obtain for consideration a set of yields from areas equal in size to sections but overlapping the normal sections in the bed. If the foregoing conjecture is correct the coefficient of variability calculated from these units of area should be lower than the coefficient of variability calculated when normal sections are used as plots. An analysis of variance based on 4 shifted plots in each of 5 beds, as shown graphically in figure 2, gave a coefficient of variability of 9.57 in contrast to the coefficient of variability of 13.6 obtained for normal sections. The conclusion, therefore, seems justified that less variability may be expected when the experiment is so arranged

FIGURE 2.—Diagram showing relative size and arrangement of plots in typical blocks. *A–D*, Plots at Arlington Experiment Farm, Rosslyn, Va.: *A*, 2-foot plots, 10 plots per block (or bed), 5 blocks (total area); *B*, 4-foot plots, 5 plots per block, 5 blocks; *C*, 6-foot plots, 3 plots per block, 5 blocks; *D*, 4-foot plots, 4 plots per block, overlapping units of filling, 5 blocks. *E* and *F*, plots at Downingtown, Pa.: *E*, 4-foot plots, 10 plots per block, 5 blocks; *F*, 8-foot plots, 5 plots per block, 5 blocks. *G–I*, plots at Coatesville, Pa.: *G*, 4-foot plots, 10 plots per block, 4 blocks; *H*, 8-foot plots, 5 plots per block, 4 blocks; *I*, 8-foot plots, 4 plots per block, overlapping units of filling, 4 blocks.

that the areas used as units for harvesting do not coincide with the areas used as units for filling the beds with compost. On an area basis the overlapping plots were 201.9 percent as efficient as the normal sections and 162.2 percent as efficient as the ½-section plots.

<center>DOWNINGTOWN, PA.</center>

The yields of 50 plots from the five experimental beds at Downingtown, Pa., are given in table 1.

When the analysis of variance summarized in table 2 is applied to these data the value of z obtained from a comparison of the variance within beds with the variance between beds is 0.47468. This is slightly less than the 5-percent point, and indicates that, unlike the Arlington Farm experiments, the precision of the experiment was not

significantly increased by the arrangement of the plots to facilitate elimination of the variability between beds. In like manner it was shown that there is no justification for arranging the experiment in Latin squares and removing variability due to columns. On an area basis, however, there can be little doubt of the advantage of using replicate small plots rather than entire beds.

The coefficient of variability, calculated from the total variance for the data obtained by harvesting plots coinciding with sections on the bed (table 2), is 19.7. When the area of the plot is doubled (fig. 2 and table 2) the coefficient of variability drops to 14.8. The lower coefficient of variability is to be expected both on the basis of the increase in the size of plot and the dissimilarity of the area used for harvesting and the area used for filling the bed with compost. However, on an area basis the double section is only 88 percent as efficient as the section plot.

<div align="center">COATESVILLE, PA.</div>

The yields of 40 plots from the 4 experimental beds at Coatesville, Pa., are given in table 1.

An analysis of variance applied to these data leads to conclusions similar to those derived from the experiments at Downingtown. Here again it is evident that small plots are preferable to entire beds on an area basis although there was no significant gain in the precision of the experiment by accounting in the analysis for the variance between beds or between columns. The coefficients of variability calculated from the total variance for single sections and double sections, respectively, were 10.6 and 7.2 (table 2). This experiment differed from the previous ones in that on an area basis the double section was slightly more efficient (108 percent) than the single section.

It should be recalled that in this experiment the units used for filling the beds with compost were double sections corresponding to plots 1 and 2, 3 and 4, 5 and 6, etc., in table 1. Theoretically the coefficient of variability should be lower if the yield data are combined so that the double sections used for plots in harvesting do not coincide with the areas used as units for filling the beds. To test this conjecture the coefficient of variability was calculated from the double sections 2 and 3, 4 and 5, 6 and 7, etc., as plots. With plots arranged in this way the coefficient of variability drops to 4.1. This corroborates the evidence in the Arlington experiments in favor of arranging the plots so as not to coincide with areas used as units in filling the beds. On an area basis double plots harvested in this manner are 308 percent as efficient as the double plots harvested from the same areas as were used for filling the beds and 334 percent as efficient as the single sections.

<div align="center">DISCUSSION AND CONCLUSIONS</div>

The foregoing experiments demonstrate conclusively that small plots are preferable to entire beds as experimental units in a mushroom house. The variance of the small plots was in all cases less than the variance of entire beds. Furthermore, the small plots permit a greater number of yield comparisons on the same area and also permit increased precision through replication and through the arrangement of the plots so as to reduce the effect of compost heterogeneity and account for the variability between beds.

The results of the uniformity trials at both Arlington and Coatesville indicate an important relationship between the arrangement of the plots and the method employed for filling the beds with compost. When areas were used for harvesting which were comparable in size to the areas used for filling the beds but which overlapped the latter areas there was markedly less variability than when the same areas were used as units for both filling and harvesting. It would seem from this that the customary method of filling the beds by emptying 10 or 12 bushel baskets of compost at a time in each section or by emptying 20 or 24 bushels into each double section induces an excessive variability in the yields from these areas. It is advisable, therefore, to arrange the plots so that they do not coincide with the units of area used for filling the beds. Probably a further gain can be made in the precision of yield tests by modifying the system of filling experimental beds in order to mix the compost and distribute it more uniformly in the beds. A practical method of doing this is to fill the beds by emptying 1 bushel of compost at a time in each section at random in the bed until every section has received the required amount of compost.

This method of mixing the compost in the bed should reduce the variability between plots on a bed, but probably would have little or no effect on the variability between beds. Therefore it is necessary to account for the variability between beds in the reduction of the data. This may be accomplished by randomizing all the treatments on each of the beds so that the beds can be considered as replicate blocks in Fisher's randomized-block system of analyzing the data. As there was no significant fertility gradient (column effect) from one end of the house to the other, randomized blocks are preferable to Latin squares in that they leave more degrees of freedom for error and thus permit a more precise test of significance.

The question of the most desirable size among small plots is some-what problematic. In all the trials the larger plots varied less than the smaller plots, so that more precision can be expected from the use of whole sections than ½-section plots, double sections than single sections, etc. But in 2 out of the 3 experiments the increase in precision with increase in plot size was not proportional to the increase in plot size. In other words, greater experimental precision was obtained for a given area by increasing the replication of small plots than by increasing the size of the plots. In practice, however, it is usually more expensive to increase replication than to increase plot size; so the experimenter must reach a compromise, depending on circumstances. In the writer's opinion sections 4 by 5 feet make satisfactory plots in a small experimental house such as the one at Arlington Farm and double sections are a good compromise for con-ventional houses. This allows the comparison of five different varieties or treatments in a single experiment if all the treatments are laid out on each of the replicate beds in accordance with the randomized-block system.

Before leaving the question of plot size, perhaps a word of expla-nation should be offered for an apparent anomaly in the results, namely, that increased precision is obtained by increasing the size of the small plots on the bed yet when entire beds are used as plots the experiments are less precise. Practical experience suggests that this is due to the fact that greater differences may be expected in the

moisture content of the compost, diseases, insect flora, and tempera-
ture from the top of the house to the bottom than from end to end.
This is substantiated by the greater variation between beds than
between columns shown in table 2.

The advisability of replicating treated plots need hardly be dis-
cussed in view of the fact, already pointed out, that on an area basis
in 2 out of the 3 uniformity trials precision was gained more rapidly
through replication than through increase in plot size. The question
at issue is how many replications are advisable under the limitations
laid down by the conditions of the experiment, the funds available,
and the structural features of the experimental house. In the
writer's opinion 5 or 6 replications are a good compromise since they
will usually enable the investigator to detect differences in yield of
approximately 10 percent, due to experimental treatment. If
greater precision is necessary it can be obtained through increased
replication, as the randomized-block plan allows as many replications
as there are beds in the house—usually 12 to 20. When different
composts are being compared it is desirable to replicate the compost
heaps in addition to replicating the plots derived from each experi-
mental compost heap.

SUMMARY

Yield data are discussed from uniformity trials in the experimental
mushroom house at Arlington Experiment Farm, Rosslyn, Va., and
in commercial houses at Coatesville and Downingtown, Pa.

These tests indicate that small plots are preferable to beds as
experimental units. Double sections containing from 40 to 48 square
feet of bed space seemed to be the most practical size of plot for
experiments in commercial houses. In one of the experiments there
was a distinct advantage in arranging the plots on the beds so as to
make it possible to account for the excess variation between beds in
analyzing the data.

A gain in precision also was apparent when test plots were so
arranged that they did not coincide with the areas used as units for
filling the beds. Therefore, a modification of the usual method of
filling beds is suggested for experimental yield tests.

At least 5 or 6 replications seem to be necessary.

MAGNESIUM, CALCIUM, AND IRON REQUIREMENTS FOR GROWTH OF AZOTOBACTER IN FREE AND FIXED NITROGEN [1]

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INTRODUCTION

Most of the investigations concerning the inorganic nutrient requirements of *Azotobacter* have not involved comparisons of growth in the presence and absence of fixed nitrogen. The majority have been chiefly qualitative studies in which free nitrogen gas only was used, and they have yielded no evidence as to whether a particular element played a necessary role in fixation or was a general growth nutrient. Burk and Lineweaver (6) [2] have recently reported an elaborate series of experiments with both macrocultures and microcultures (Erlenmeyer and Warburg technic) in which calcium (or strontium) was the only metallic ion found to be specifically required in the catalysis of the nitrogen-fixing process at concentrations greater than 0.01 millimolal. Magnesium and iron (7) were found to be highly stimulating to the growth process in both free and fixed nitrogen over the respective concentration ranges 0.1 to 3 millimolal and 0.001 to 0.015 millimolal, but no studies were carried out at lower concentrations to ascertain whether these elements were strictly essential.

The present work has extended these investigations in a quantitative manner, with particular reference to the absolute growth requirements. Considerably modified cultural conditions have been employed. The experiments have lasted several days or weeks, instead of 6 to 48 hours, thereby permitting maximum growth. The nutrient media have been maintained at the most recently determined optimum concentrations with respect to all known nutrient requirements except the element or elements under consideration.

REVIEW OF LITERATURE

According to Buchanan and Fulmer (5), magnesium is essential to the growth of many, though not all, micro-organisms. Linossier (22) has shown that it is required for the development of *Oidium lactis*, Buromsky (11) that it is essential for the growth of *Aspergillus niger* and not replaceable by calcium, and Lockemann (23) and Frouin and Guillaumie (13) that it is not replaceable by calcium, strontium, or any of the rare earth metals in the case of the tuberculosis bacillus. Frouin and Ledebt (14) have shown magnesium to be replaceable by certain of the rare earths for the production of pigment by *Bacillus phocaneus*. Many others report stimulating action upon microorganisms at low concentrations of magnesium, with toxicity as the concentration is increased to 100 to 500 millimolal; however, essentialness at very low concentrations has rarely been definitely determined.

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Rippel and Stoess (*27*) concluded from experiments with a variety of bacteria and fungi, as well as from a review of the literature, that calcium is not a generally indispensable nutrient for micro-organisms but is occasionally necessary for some special physiological process, such as nitrogen fixation by *Azotobacter*. On the basis of his comprehensive experiments concerning the calcium requirements of living forms, Loew (*24*) concluded that most lower organisms, in contrast to the algae and higher organisms, rarely contain calcium.

Krzemieniewska (*21*), who has made one of the few quantitative studies of the inorganic nutrient requirements of *Azotobacter*, found that 0.16 millimolal of magnesium and 0.12 millimolal of calcium were necessary for maximum growth, whereas zero concentrations of either substantially prevented all growth and nitrogen fixation. The experiments were carried out in free nitrogen only, however, so that a comparison of the action of either element on the growth and fixation processes was not possible. Schröder (*29*) found calcium not to be essential for *Azotobacter* growth in fixed nitrogen, but stimulating, and concluded that this effect was due more to a neutralizing action than to any specific effect of the calcium ion. Stapp and Ruschmann (*30*) found little or no beneficial effect of calcium in a medium containing nitrate.

Very little work has been carried out on the iron requirements for bacterial growth because the limiting concentration range is usually about one-thousandth that of elements such as magnesium or calcium, and most culture media, especially those containing organic nutrients, generally contain sufficient impurity to produce maximum growth. A brief review of the most pertinent previous investigations on general bacterial growth is given by Buchanan and Fulmer (*5, p. 411*). Previous work with respect to *Azotobacter* has been reviewed elsewhere (*7, pp. 441–442*).

METHODS

Several media were employed, termed "basal" (A), "customary" (B), "altered" (C), "humate-free" (D), and "charcoal-treated" (E). The basal medium A consisted of 0.64 g (3.68 millimolal) K_2HPO_4; 0.16 g (1.18 millimolal) KH_2PO_4; 0.2 g (0.81 millimolal) $MgSO_4 7H_2O$; 0.05 g (0.29 millimolal) $CaSO_4 2H_2O$; 0.2 g (3.42 millimolal) NaCl; 0.5 mg (0.009 millimolal) Fe as synthetic humate iron [3]; and 20 g (55.5 millimolal) Mallinckrodt's sucrose crystals per liter. This medium was used as a standard control in most of the work. It provided a heavy growth of *Azotobacter* (20 mg organic nitrogen per 100 cc) and did not precipitate upon being sterilized. Customary medium B, employed previously (*6, 7*), contained 1 percent glucose instead of 2 percent sucrose, no humate iron, and slightly more calcium and phosphate. Altered medium C contained more or less added Ca or Mg than basal medium A; it precipitated upon standing if the Mg content were lowered to 0.04 millimolal or the Ca or phosphate content increased 20 percent or more. Humate-free medium D consisted of basal medium A with no Fe added as humate iron. Charcoal-treated medium E was prepared by shaking 1 l of humate-free medium D with 75 g of purified Baker's animal charcoal, allowing to stand for several days, and filtering. Humate iron was added to the filtrate before use in order to insure optimum iron for growth.

[3] For method of preparation see the following publication: HORNER, C. K., BURK, D., and HOOVER, S. R. THE PREPARATION OF HUMATE IRON AND OTHER HUMATE METALS. Plant Physiol. (In press.) 1934.

Preliminary purification of the charcoal in order to remove considerable amounts of adsorbed gases and other impurities was accomplished by leaching 3 to 6 times at intervals of several hours with equal weights of 6 N HCl and finally washing on a Buchner filter with dilute alkali and then distilled water until the wash water was neutral.

Fixed nitrogen when employed was added at the rate of 100 to 200 mg (7.1 to 14.2 millimolal) per liter, as KNO_3, NH_4Cl, or urea.

Erlenmeyer or Florence flasks of 150 to 250 cc capacity containing 15 to 25 cc of nutrient solution and plugged with cotton were employed as culture flasks. Sterilization was accomplished by autoclaving for 10 minutes at 15 pounds pressure, 20 percent extra distilled water being added to each flask to make up for loss by evaporation. The culture flasks were inoculated with 3 drops of culture grown in aeration bottles containing 100 cc of customary medium. In the experiments where it was desired to minimize as much as possible any Ca or Mg carried over by the inoculum, heavy 2- to 7-day cultures were diluted 50 to 100 times with sterile medium free from these elements. The cultures were incubated in duplicate or triplicate at 28° to 30° C. for 2 to 10 days, occasionally longer, with frequent observations.

Growth was measured turbidimetrically with a Bausch & Lomb nephelometer, corrections being made for any significant original turbidity in uninoculated cultures (cultures of low Mg content). The original and final pH values were measured colorimetrically by using the various La Motte indicators and standards. The original pH of all media was 7.0 ± 0.2. In general, the pH values tended to decrease a maximum of 1 to 2 units with increase in growth, except in the case of cultures with nitrate, where the values often increased. The buffering capacity of the medium was substantially independent of the Ca or Mg concentration, being controlled chiefly by the phosphate concentration. In general, the sugar was rarely entirely consumed by the end of an experiment.

The organism employed was *Azotobacter vinelandii*, capable of very active nitrogen fixation and long in use at this laboratory. According to the recent work of Kluyver and van Reenen (*18*), this species is the common motile form of *Azotobacter*, of relatively small size, occurring in soils. It produces a green fluorescent pigment, and at times assumes either a lighter, yellow, or darker, pink or purple, tinge, especially in the presence of molybdenum.

EXPERIMENTAL RESULTS

MAGNESIUM AND CALCIUM

Tables 1, 2, and 3 show fair examples of the relative growths obtained with *Azotobacter* with a wide variety of Mg and Ca concentrations under otherwise approximately optimal conditions. The ratios of growths in altered media C to those in basal medium A are given as the most direct means of comparing the results in the different experiments at various stages of growth and with the three forms of nitrogen employed. Table 1 shows the effect upon growth of lowering either Mg or Ca while the other element is kept constant at the usual concentration; the results with varying Mg in the 10-day experiment are plotted in figure 1.

In general, the fraction of normal growth at any concentration of Mg is roughly the same in free or fixed nitrogen, although the actual

growth may be 2 to 4 times as great in the latter. The fraction tends to become smaller with the duration of the experiment and extent of growth, as would be expected if Mg were an essential cell nutrient. In other experiments lasting 30 to 40 days, cultures without Mg were only about one-hundredth as turbid as the basal medium controls.
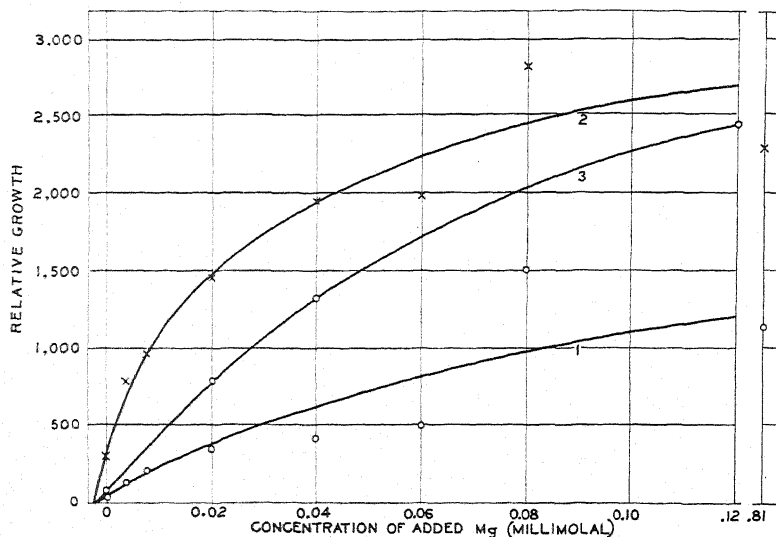
FIGURE 1.—Growth of *Azotobacter* in free and fixed nitrogen as a function of magnesium concentration Curves 1 and 2, growth (turbidity) in N_2 and nitrate, respectively, 10-day experiment (table 1). Curve 3, growth (nitrogen fixed) in N_2, 12-day experiment, data of Krzemieniewska (*21*) (ordinate scale different from that for curves 1 and 2).

The growth of *Azotobacter*, independently of the source of nitrogen, decreases from normal at 0.05 to 0.1 millimolal Mg to one-fiftieth to one-hundredth of normal at 0.002 millimolal Mg (estimated maximum impurity in basal medium and inoculum).

TABLE 1.—*Effect of Ca and Mg concentrations upon Azotobacter growth and fixation*

Ca	Mg	Growth (turbidity) ratio, [a] $\frac{\text{altered medium C}}{\text{basal medium A}}$ in—					
		Experiment 1, 3 days			Experiment 2, 10 days		
		N_2 (air)	KNO_3	Urea	N_2 (air)	KNO_3	Urea
Milli-molal	*Milli-molal*						
[b] 0.29	[b] 0.810	1.000	1.000	1.000	1.000	1.000	1.000
.29	.081	1.000	.573	.940	1.320	1.240	1.300
.29	.061	.356	.382	.658	.450	.873	1.270
.29	.040	.339	.339	.439	.367	.855	.795
.29	.020	.339	.329	.387	.306	.640	.306
.29	.008	.319	.329	.327	.181	.423	.183
.29	.004	.319	.267	.204	.122	.350	.144
.29	.000	.257	.114	.084	.037	.141	.066
.15	.810	.808	.958	.800	.718	.953	1.120
.06	.810	.628	1.040	.648	.768	1.050	1.220
.03	.810	.532	.840	.550	.568	1.295	1.190
.006	.810	.172	.710	.523	.437	.940	1.060
.00	.810	.130	.545	.470	.295	.606	1.190

[a] Relative growths in basal medium A—N_2: NO_3:urea (3 days) = 1:3.7:3.7; (10 days) = 1:2.0:4.1.
[b] Basal medium A.

TABLE 2.—*Effect of variable Ca-Mg ratios upon Azotobacter growth and fixation*

Ca	Mg	Ca/Mg	Growth (turbidity) ratio,[a] $\frac{\text{altered medium C}}{\text{basal medium A}}$ in—								
			Experiment 1, 4 days			Experiment 2, 7 days			Experiment 3, 10 days		
			N_2(air)	KNO_3	Urea	N_2(air)	KNO_3	Urea	N_2(air)	KNO_3	Urea
Milli-molal	*Milli-molal*										
0.290	0.0	∞	0.162	0.628	0.230	0.101	0.315	0.093	0.032	0.332	0.073
.029	.0	∞	.085	.424	.280	-----	-----	-----	.069	.202	.085
.012	.0	∞	-----	-----	-----	.016	.175	.074	-----	-----	-----
.290	.081	3.58	1.220	.695	1.565	-----	-----	-----	.981	.935	1.010
.012	.016	.75	-----	-----	-----	.015	.589	.448	-----	-----	-----
.006	.008	.75	-----	-----	-----	.018	.438	.214	-----	-----	-----
[b].290	[b].810	.36	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
.087	.240	.36	.918	.742	1.190	-----	-----	-----	.705	.565	1.000
.029	.081	.36	.166	.935	.878	-----	-----	-----	.158	.935	.885
[c].004	[c].028	.14	.130	.653	.638	.009	.667	1.000	.016	.556	1.030
.029	.81	.036	.695	.910	.878	-----	-----	-----	.276	.764	.788
.029	2.43	.012	.855	1.125	.553	-----	-----	-----	.329	.658	.963
.029	4.86	.006	.618	1.125	.807	-----	-----	-----	.287	.633	1.030
.0	4.86	.0	.297	.963	.745	-----	-----	-----	.158	.568	.980
.0	2.43	.0	.364	1.030	1.090	-----	-----	-----	.333	.475	.878
.0	.81	.0	.173	.910	1.150	.164	.642	.972	.142	.424	1.050
.0	.081	.0	.067	.963	.807	-----	-----	-----	.037	.742	.953
.0	.016	.0	-----	-----	-----	.020	.547	.396	-----	-----	-----
.0	.0	-----	.068	.454	.214	.013	.317	.108	.010	.263	.089

[a] Relative growths in basal medium A—N_2:NO_3:urea (4 days) = 1.0:2.7:4.8; (7 days) = 1.0:1.4:2.1; (10 days) = 1.0:0.9:1.5.
[b] Basal medium A.
[c] Charcoal-treated medium E.

TABLE 3.—*Influence of Ca in medium and inoculum upon growth in free and fixed nitrogen*

Nitrogen source	Ca in medium	Ca in inoculum	Growth (turbidity) in		
			2 days	3 days	6 days
N_2	[a] +	[a] +	-----	37	123
	+	-	-----	36	107
	[b] -	[b] +	-----	7	24
	-	-	-----	6	26
KNO_3	+	+	58	121	234
	+	-	56	92	227
	-	+	25	65	286
	-	-	25	75	149
Urea	+	+	44	212	484
	+	-	34	172	374
	-	+	43	209	472
	-	-	29	121	455
NH_4Cl	+	+	88	166	231
	+	-	52	141	231
	-	+	81	83	123
	-	-	60	100	140
Average of KNO_3, urea, and NH_4Cl	+	+	63	167	316
	+	-	47	135	277
	-	+	50	179	293
	-	-	38	99	248

[a] Basal medium A.
[b] Altered medium C (basal medium A without Ca). KNO_3 present in Ca-free medium used to prepare inoculum; such inoculum grown for 3 transfers previous to use.

When the Ca of the medium is lowered, a different relationship is seen to exist. Whereas the growth in fixed nitrogen may be decreased a maximum of 50 percent, that in free nitrogen is lowered 70 to 90 percent when but traces of Ca are present. Ca deficiency is more pronounced in the early stages of growth in both free and fixed

nitrogen. As growth progresses the need for Ca in fixed nitrogen is practically nullified; and, whereas a definite effect remains in free nitrogen, after a sufficient time (30 to 40 days) mere traces of Ca will permit 25 to 50 percent of normal growth. This substantiates the conception that Ca acts in a truly catalytic role in nitrogen fixation; only traces are absolutely necessary provided sufficient time is allowed, but relatively high concentrations are required to produce a maximum rate of fixation. The Ca needed for normal maximum growth in free nitrogen is 0.15 to 0.30 millimolal or practically saturation (0.36 millimolal), and this range appears to hold also in the early stages of growth in fixed nitrogen, but decreases after several days to less than 0.01 millimolal in nitrate and presumably to zero in urea (and ammonia).

The decrease with the duration of the experiment in the Ca requirements for growth in fixed nitrogen is indicated in table 3. The culturing of the inoculum for several previous transfers in Ca-free medium does not enable the organisms to become adapted to such a medium since somewhat less growth is obtained, as compared to inoculum grown in a medium containing calcium. This is best seen in the "average" values and tends to indicate a very small but definite Ca requirement in fixed nitrogen for entirely maximum, as distinguished from approximately maximum, growth. It is seen that the Ca requirement in free nitrogen remains considerable even after 6 days, the ratio of growth in its presence and absence (except for impurities) being 6 to 1 at 3 days and 4½ to 1 at 6 days.

In table 2 are given the growth ratios with a variety of Ca/Mg ratios principally under suboptimal concentrations of both elements. The most definite results are obtained with the older cultures. It is seen that little if any effect can be attributed to the Ca/Mg ratio, as has been reported frequently with supraoptimal concentrations with various organisms in connection with antagonism (_5, pp. 282–284, 356–359_). The independent effect of each element can be analyzed. Varying the Ca in the absence of Mg has very little effect in any case, since the lack of Mg is already limiting the growth almost completely. The two 0.75 Ca/Mg ratios show no significant difference in N_2, the Ca and Mg both being highly limiting; in fixed nitrogen the decrease corresponds to the Mg decrease. The three 0.36 ratios, with widely varying concentrations of the two elements, show definite decreases of growth in N_2 (due to calcium deficiency), but relatively little in fixed nitrogen. Keeping the Ca constant at 0 or 0.029 millimolal and increasing the Mg sixfold from 0.81 to 4.86 millimolal causes no consistent significant change in the amount of growth. As indicated previously, toxic effects are obtained at concentrations much greater than 4.86 millimolal (6 "customary").

The charcoal treatment of basal medium A was developed to remove, if possible, some nutrient or nutrients specifically required in growth or fixation, either in large or very small concentration. Hopkins (_17_) used this method (without the charcoal purification) to remove traces of iron from dissolved sugar. As shown in table 2 (Ca/Mg = 0.14), the effect of the treatment can be attributed almost entirely to the Ca and Mg removed; the Ca was found upon analysis, by an oxalate precipitation method, to have been reduced to about one-seventieth, and the Mg, by an ammonium phosphate precipita-

tion method, to one-thirtieth of the original concentrations. The Mg retained is still sufficient to permit 50 to 100 percent normal growth in fixed nitrogen, whereas in free nitrogen the combined Mg-Ca deficiency permits but slight growth, owing chiefly to lack of Ca and secondarily to lack of Mg; the effect of Mg deficiency is enhanced at low Ca. The average growth ratios in the charcoal-treated medium E for seven different 6- to 10-day experiments were: N_2, 0.015; KNO_3, 0.595; urea, 0.715. As may be seen in table 2, these growth ratios are approximately those obtained when the same order of Ca and Mg concentrations are added intentionally in making up altered media.

The preponderant influence of Ca-Mg deficiency in the charcoal-treated medium E was also demonstrated by determining the effect of adding thereto the different basal medium A salts separately. The KH_2PO_4, K_2HPO_4, and NaCl caused substantially no improvement. Humate iron was normally added to all charcoal-treated medium E, but special experiments involving its omission showed that it likewise was not beneficial. Table 4 shows the effect of adding Mg and Ca separately and together to the charcoal-treated medium E. The most striking results, as would be expected, are observed in free nitrogen. Ca alone increases the growth about 7 times, Mg about 18 times, and both together about 45 times, or to within 25 percent of that in basal medium A. The fact that Ca and Mg together did not cause complete recovery indicates that some other slight inhibiting factor may be involved in the charcoal treatment, but a 10- to 30-percent variation can be expected in the methods of culturing and measurement employed. Since the deficiency in the case of fixed nitrogen is not sufficient to cause more than this amount of inhibition, no definite beneficial effect of the added Ca and Mg can be detected. Neither the Ca nor the Mg alone is sufficient to return the growth to normal since the other element is always deficient. When either Mg or Ca solutions (of basal medium A concentration) are treated with charcoal and added to the other salts untreated, both media so obtained show growth inhibition, as would be expected. This inhibition is overcome by adding Mg or Ca, respectively. Similar treatment of phosphate or NaCl solutions yields no inhibition. It may be stated also that the extent of reduced growth obtained in charcoal-treated medium E was independent of the concentration of Fe added and also the amount or age of inoculum. Finally, sugar charcoal as compared with animal charcoal yielded no growth reduction; in fact it was observed to provide iron in media deficient in this element.

TABLE 4.—*Growth recovery in charcoal-treated medium E upon addition of Mg or Ca*

Medium	Growth (turbidity) ratio,[a] $\frac{\text{altered medium C}}{\text{basal medium A}}$ when source of nitrogen was—		
	N_2 (air)	KNO_3	Urea
Basal A	1. 000	1. 000	1. 000
Charcoal-treated E	. 016	. 787	. 708
Charcoal-treated E+0.29 millimolal Ca	. 115	. 805	[b]. 565
Charcoal-treated E+0.81 millimolal Mg	. 297	[b]. 595	. 845
Charcoal-treated E+0.29 millimolal Ca and 0.81 millimolal Mg	. 732	. 787	. 808

[a] Relative growths in basal medium A—N_2:NO_3:urea (6 days) = 1.0:1.0:1.7.
[b] Low.

The apparent essentialness of Mg for *Azotobacter* growth, as observed in the preceding experiments, is almost certainly a true magnesium requirement. The Baker's $MgSO_4.7H_2O$ employed was examined spectroscopically.[4] Only sodium was found in considerable amount, with definite but faint indication of aluminum, copper, calcium, iron, lithium, and silicon, and possibly vanadium and boron (no potassium). All these elements, and others, were tested for their effect in either charcoal-treated medium E or basal medium A containing no Mg. The following metal ions were added to charcoal-treated medium E: Cu, Mn, Ni, Co, Al, Zn at 0.1 p.p.m.; Mo, Si, Ti, Cr at 1 p.p.m.; V, Mn, B, Li, and Ba at 0.1 and 1 p.p.m.; 20 and 200 p.p.m. of natural humic acid, a material which undoubtedly contains traces of all basic elements normally found in soil; and also 10 p.p.m. coenzyme R (1). No increase in growth was observed with any of these. In the magnesium-free medium the following metals were tested: V, B, Mo, and Li at 2.5 and 10 p.p.m.; Ba and Sr at 2.5, 10, 25, and 50 p.p.m.; and Mn at 0.5, 2.5, 10, and 12.5 p.p.m. Mn, Ba, and Sr appeared to be slightly beneficial. The other elements yielded no increase in growth. Mg as such is therefore essential with no element able to act very efficiently in place of it. No stimulation in basal medium A resulted from 0.1 and 1 p.p.m. Cu, Al, Ni, and Mn, but stimulation was often increased $1\frac{1}{2}$ to 3 times by either V (VCl_3, Na_3VO_4, or $K_2V_4O_9$) or Mo ($NaMoO_42H_2O$) at 1 p.p.m., in agreement with previous findings of Bortels (3, 4), Burk and Lineweaver (6), Birch-Hirschfeld (2), Schröder (29), Kluyver and Van Reenen (18), and Konishi and Tsuge (19).

<center>IRON</center>

The problem of demonstrating a definite iron requirement usually necessitates purification of the medium rather than the addition of iron, owing to the low values involved. The phosphate-adsorption method reported by Hopkins (17) in connection with the green alga *Chlorella*, and the carbonate treatment described by Steinberg (31) in connection with the bread mold *Aspergillus*, for removing traces of Fe were found to be incapable of causing any Fe deficiency for *Azotobacter* in basal medium A without humate iron (medium D). There still remained after treatment 0.001 to 0.003 millimolal Fe. The modified Hopkins' charcoal treatment employed for removal of Mg and Ca masked any possible removal of Fe. Ruhland's (28) method of autoclaving and filtering a nutrient solution composed only of inorganic nutrients was found to lower the Fe impurity of the inorganic salts of basal medium A but could not be employed for sugar purification. Several sugars were, therefore, obtained, which when analyzed by a method previously described (7, *table 1*), showed a varying and relatively low Fe content, as follows: Difco dextrose, 4×10^{-4} percent; Merck's dextrose, 6×10^{-5} percent; Baker's sucrose, 6×10^{-5} percent; and Mallinckrodt's sucrose, 9×10^{-6} percent. The inorganic basal medium A (without humate iron or sugar) contained before and after autoclaving and filtering while hot 2.6×10^{-5} millimolal and 1.7×10^{-5} millimolal Fe, respectively. It was possible to obtain 8 Fe concentrations ranging from 5×10^{-5} millimolal to 4.5×10^{-4} millimolal by employing each of the 4 sugars at 13.9 millimolal (0.5 percent) together with 41.6 millimolal (1.5 percent) of the

Mallinckrodt's (very low iron) sucrose, basal inorganic medium A being used with and without 5×10^{-5} millimolal humate iron (Fe impurity in synthetic humic acid with no Fe intentionally added); the lowest concentration was obtained by using 55.5 millimolal (2 percent) Mallinckrodt's sucrose in inorganic medium previously autoclaved and filtered. Maximum iron was assured by adding 1.8×10^{-3} millimolal humate iron to some of the cultures.

Figure 2 shows the relative growths obtained with these varying Fe concentrations. The curves definitely approach zero growth at the lowest concentrations, whereas the sugar, Difco dextrose, with the maximum Fe impurity, 4×10^{-5} millimolal, yields practically normal growth. The concentration of Fe yielding maximum growth increases somewhat with age of culture (curves 1 and 1a, 2 and 2a), as was shown previously in a different manner in connection with humic
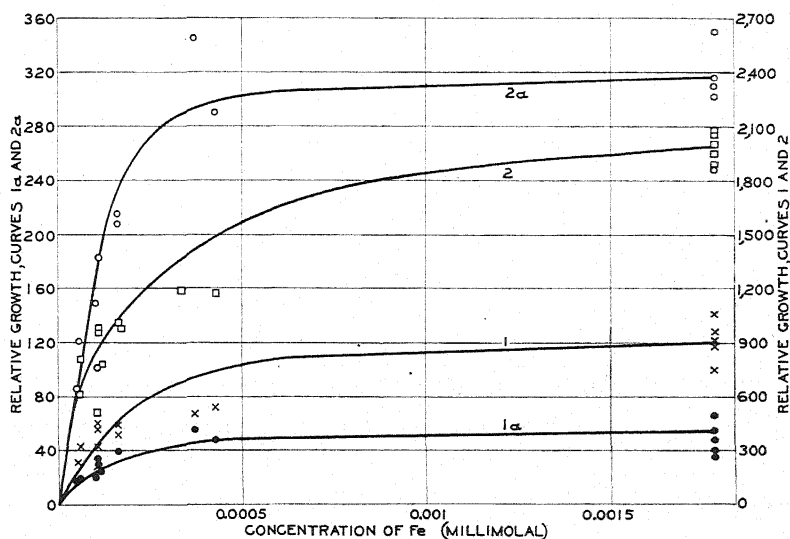
FIGURE 2.—Growth of *Azotobacter* in free and fixed nitrogen as a function of iron concentration: Curves 1 and 2, growth (turbidity) in N_2 and nitrate, respectively, 9-day experiment; curves, 1a and 2a, 2-day experiment.

acid stimulation (*8, p. 458, fig. 2*). At any given age of culture approximately the same differences in amount of growth in free and fixed nitrogen are obtained at all iron concentrations (table 5), indicating that, as determined by this method, there was no specific fixation effect of Fe deficiency, such as was obtained so definitely in the case of Ca. The phenomenon of the growth in free nitrogen approaching that in fixed nitrogen, after a prolonged period of time, is shown by the "average" values, table 5, and by comparing the ordinate ranges between curves 1 and 2 and curves 1a and 2a. This phenomenon is commonly observed and presumably bears no direct relation to Fe requirements, but to limitations by other factors (*8, pp. 455–462*). Ruhland (*28*) has reported a limiting Fe concentration range for the autotrophic hydrogen organism *Bacillus pycnoticus* which is essentially the same as that demonstrated for *Azotobacter*, no growth being obtained at 10^{-5} millimolal and maximum growth occurring by 1.2×10^{-3} millimolal.

TABLE 5.—*Ratio of growth in fixed and free nitrogen at different iron concentrations*

Concentration of Fe (millimolal)	Growth (turbidity) ratio, $\frac{KNO_3}{N_2}$ for experiment—			
	No. 1 [a]		No. 2	
	2 days	9 days	3 days	5 days
4.8×10^{-5}	5.0	2.7		
5.9×10^{-5}	6.7	2.6		
1.01×10^{-4}	5.5 {3.4, 6.0, 7.0}	2.3 {2.3, 2.2, 2.4}		
1.1×10^{-4}	7.0	2.4		
1.6×10^{-4}	5.6 {5.4, 5.7}	2.4 {2.3, 2.5}		
4.1×10^{-4}	6.2	2.3		
4.6×10^{-4}	6.1	2.2		
1.8×10^{-3}	6.6 {5.4, 8.0, 3.8, 7.3, 8.7}	2.2 {2.0, 2.1, 2.0, 2.3, 2.6}		
1.8×10^{-2}	5.7 {5.8, 7.4, 2.7, 5.6, 7.1}	2.1 {1.6, 2.0, 2.6, 1.9, 2.4}		
2.5×10^{-5}			4.6	4.3
3.6×10^{-5}			3.0	3.5
8.1×10^{-5}			5.7	2.6
3.8×10^{-4}			4.3	3.3
1.8×10^{-2}			4.5 {4.3, 4.3, 5.3, 4.2}	3.1 {3.1, 2.9, 2.0, 4.4}
Average	6.1	2.3	4.4	3.4

[a] Compare fig. 2, p. 989.

COMPARISON OF ABSOLUTE ELEMENT REQUIREMENTS

Comparing the concentrations of essential elements producing half maximum growth ($C_{G/2}$) is as convenient, and more accurate, than comparing those required for optimum growth, under variable experimental conditions. Table 6 gives $C_{G/2}$ values for Ca, Mg, and Fe under various conditions of age of culture and nitrogen source, and also those for Ca and Mg determined from Krzemieniewska's data (21). These values have been determined graphically from curves such as those shown in figure 1 for Mg and in figure 2 for Fe. The values for Mg and Fe do not vary significantly with respect to nitrogen source, being essential elements in any case, whereas the nonessentialness of Ca in fixed nitrogen is strikingly shown by the lack of any definite positive value, as compared with 2×10^{-2} to 5×10^{-2} millimolal in free nitrogen. $C_{G/2}$ for Fe is about one five-hundredth of that for Mg or Ca in free nitrogen.

In general, $C_{G/2}$ should decrease with age or heaviness of cultures if the element is utilized relatively more efficiently at lower concentrations by being employed over and over again in a catalytic manner. It should increase if the element is consumed and prevented from further functioning. As can be seen from table 6, there are no changes in $C_{G/2}$ for any of the elements, great enough to distinguish conclusively between catalysis or consumption or a combination of both. A tendency appears for a decrease with Mg and Ca and an increase with Fe, and earlier evidence with Ca and Fe supports this interpre-

tation. $C_{G/2}$ probably varies relatively little for growths ranging from 2 to 20 mg organic nitrogen per 100 cc (light to very heavy growths, about 20 to 200 mg dry matter per 100 cc); it should be a fairly characteristic constant. This is important in connection with the basal medium A employed, since normally Mo and V were not added, although in experiments involving very heavy growth they could cause definite increases in growth.

TABLE 6.—*Approximate concentrations of calcium, magnesium, and iron required for half maximum growth of Azotobacter using various sources of nitrogen*

Element	$C_{G/2}$, the concentration (millimolal) yielding half-maximum growth when source of nitrogen was—							
	N₂ (air)		KNO₃		Urea		N₂ (Krzemieniewska (*21*))	
	2–3 days	9–10 days	2–3 days	9–10 days	2–3 days	9–10 days	5 days	12–15 days
Ca	4×10^{-2}	2×10^{-2}	$^a0$–2×10^{-4}	$^a0$–2×10^{-4}	$^a0$–2×10^{-4}	$^a0$–2×10^{-4}	8×10^{-2}	5×10^{-2}
Mg	6×10^{-2}	4×10^{-2}	6×10^{-2}	2×10^{-2}	5×10^{-2}	3×10^{-2}	5×10^{-2}	4×10^{-2}
Fe	1.1×10^{-4}	1.4×10^{-4}	1.1×10^{-4}	1.6×10^{-4}				

^{a} Maximum Ca impurity in medium and inoculum.

Krzemieniewska (*21*) reported that potassium, sulphur, and phosphorus were required for *Azotobacter* growth in relatively large concentrations, the first-named at about that found for Ca and Mg, and the latter at a value 3 to 10 times as great. Vogel (*32*) and Stapp and Ruschmann (*30*) report, however, that the addition of potassium to media was not required. Similarly the present writers, and Stapp and Ruschmann (*30*), have found that the traces of sulphur occurring as impurities will yield at least 50 to 75 percent of maximum growth. The phosphorus requirements have been generally assumed to be relatively high; Burk and Lineweaver (*6*) have indicated that 0.1 millimolal is sufficient.

DISCUSSION

The experiments of long duration reported above are seen to confirm the previous observations (*6*) involving experiments essentially of short duration concerning the catalytic effect of Ca upon the nitrogen-fixation process of *Azotobacter*. The small concentrations of Ca studied in the present work yield still more evidence that Ca acts in a truly catalytic role. The relatively large concentrations emphasized previously (0.10 to 0.30 millimolal) applied to obtaining the maximum rate of fixation by young cultures, rather than to the maximum amount of growth after a long period of time. The apparent stimulation of growth in fixed nitrogen by Ca is much lessened, if not upon occasion entirely eliminated, as the duration of the experiment is increased.

The essentialness of Mg for the growth of *Azotobacter* is much more evident from the experiments of long duration, with the other inorganic nutrients at normal concentrations, than in the experiments of Burk and Lineweaver (*6*) where 10 times the customary diluted medium was chiefly employed. Whereas some growth may occur in free nitrogen with less than 2×10^{-3} millimolal Mg (estimated maxi-

mum Mg impurity) in the first 24 to 48 hours, surpassing that obtained with a medium containing 0.81 millimolal Mg, but no Ca, growth in the former practically ceases after a few days, whereas in the latter a definite increase occurs. In this case the Ca is being used continuously at very small concentrations.

As mentioned above, and observed in a former paper (*6, p. 164*, "pseudo-Ca-Sr-effect"), Mg appears to possess at times a somewhat, greater relative beneficial effect upon growth in free nitrogen than in fixed nitrogen, particularly at low concentrations of Ca. For the present, this is still interpreted as involving no specific influence upon the fixation process proper, but probably making any Ca present in the cells more available, perhaps merely more soluble. In this connection, Mg has been observed to interfere markedly with Ca precipitation by oxalate, and also, as mentioned above, serves to maintain a clear nutrient solution by increasing the solubility of calcium phosphate. This effect upon Ca solubility would scarcely be related to the chief effect of Mg in the growth process, since Ca is substantially unnecessary there.

Haines (*16*) has reported a somewhat parallel relationship with Ca and Mg between the production of bacterial gelatinase and growth with five different micro-organisms. Ca alone promotes the formation of the enzyme while permitting but poor growth; Mg alone yields good growth but substantially no gelatinase. The best effect is obtained in the presence of both elements, probably owing in part to the influence of growth itself upon the protease production. In a previous work, Haines (*15*) reported a similar effect with the enzyme attacking caseinogen. He suggests that future work might show salts of Ca to be essential for the formation of proteases in general. Although this view might appear contradictory to Rippel and Stoess' (*27*) conclusion as to the general unessentialness for Ca for microorganisms, since the majority probably produce proteases, it suggests in connection with the present work with *Azotobacter* that Ca when essential for organisms is a constituent of some particular enzyme. Thus, Nakamura (*26*) has found that Ca is an essential activator for amylase. The concentration of Ca has recently been shown, however, to have no influence on two specific aspects of fixation by *Azotobacter*, namely, the pH limit at 6, and the Michaelis dissociation constant or nitrogen pressure at which half maximum velocity of fixation is obtained (*9*).

Mg may also act specifically as a constituent of certain frequently occurring enzymes, as well as in some general phenomenon such as permeability. Lohmann (*25*) has found that Mg is an essential constituent of the coenzyme for lactic acid formation in muscle and for cozymase in yeast, and Erdtman (*12*) has found that it is necessary for liver phosphatase. The general utilization of Mg in oxidative processes is improbable, however, because of their diversity in the various organisms. Kruse, Orent, and McCollum (*20*) have shown that Mg deficiency in young rats leads to death and that the most striking blood change is a disturbance of the lipoid distribution. This finding may disclose upon further investigation that Mg is concerned generally in some phase of lipoid behavior.

The observation that the iron requirement of *Azotobacter*, as measured by final amounts of growth obtained with the Erlenmeyer technic, is substantially independent of the source of nitrogen con-

firms the previous finding (8, *pp. 465, 482, 486; 10, p. 523*) that in experiments of this type the stimulation caused by iron supplied to customary medium, as humate or otherwise, is approximately the same in free and fixed nitrogen; that is, iron does not normally appear to influence directly or specifically the availability of either of these sources of nitrogen, the one more than the other. The very much lower concentrations of Fe as compared to Ca required in free nitrogen make a specific fixation requirement somewhat more difficult to determine by the methods here employed. In earlier papers reference was made (7, *pp. 427, 447; 8, p. 465 and table 6; 10, p. 523*) to unpublished work on the apparent specific effect of iron in fixation under certain particular conditions, obtained with the Warburg technic, where the velocity constants as distinguished from the final amounts of growth were observed. This apparent specific effect of iron has now been definitely determined to be due to an exceedingly small molybdenum impurity in the iron. The molybdenum is effective at a concentration of only 1 to 100 mg [4] per 1,000,000,000,000 mg medium (1 to 100 parts per trillion, or 10^{-11} to 10^{-9} molal Mo.)

The amounts of iron required for maximum growth of *Azotobacter* as determined in the experiments reported here are approximately the same as those indicated in previous work (7), namely, 0.0004 to 0.01 millimolal (0.02–0.5 p.p.m.). In the experiments described in table 5 and figure 2, the concentration 0.018 millimolal as compared to 0.0018 millimolal gave growths 10 and 22 percent higher, on an average, at 2 and 9 days, respectively. No similar comparison of $C_{G/2}$ values is possible, since it is only in the present work that the low concentrations of Fe have been studied.

SUMMARY

The magnesium, calcium, and iron requirements for growth of *Azotobacter vinelandii* in the presence of free and various forms of fixed nitrogen have been investigated quantitatively, and brief reference made to other inorganic elements. Various types of media were employed. In comparison with work reported previously, the experiments were of relatively long duration (2 to 10 days), and the growths obtained were very heavy (20 to 200 mg dry matter per 100 cc).

The concentrations of the respective elements yielding half maximum growth ($C_{G/2}$) have been determined as the most accurate means of making various comparisons. In these experiments $C_{G/2}$ was relatively independent of duration of experiment and extent of growth. $C_{G/2}$ for Ca is 2–5 $\times$ 10^{-2} millimolal in free nitrogen, and negligible (0–2×10^{-4} millimolal) in fixed nitrogen, such as nitrate, ammonia, or urea; this difference confirms previous findings concerning the specific role of calcium in the nitrogen-fixing process. With Mg and Fe, $C_{G/2}$ is independent of the source of nitrogen, being 2–6 $\times$ 10^{-2} and 1.1–1.6×10^{-4} millimolal, respectively. The essential role of both Mg and Fe in growth is indicated by an approach to zero growth at the lowest concentrations. No specific requirement of Fe (or Mg) in fixation was evident in experiments of the type here employed, confirming previous findings that normally humate iron exerts the same stimulation in free and fixed nitrogen.

The concentrations of Mg, Ca (in free nitrogen), and Fe required for maximum growth are 0.05–0.1 millimolal, 0.1–0.3 millimolal, and 0.0004–0.001 millimolal, respectively. The requirement for P appears to be 0.1 millimolal, and for S, K, Mo, and V equal to or less than that for Fe.

Mg could not be replaced in the growth process by Cu, Mn, Ni, Co, Al, Zn, Ca, Sr, Ba, Mo, Si, Ti, Cr, V, B, or Li applied in various concentrations.

In ascertaining the very low Fe requirement, adsorption methods involving charcoal, calcium carbonate, or calcium phosphate did not suffice to free the medium from iron. It was necessary to select sugars with different and very low amounts of iron. This method should be useful in connection with various elements similarly needed in traces in general bacterial growth.

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THE NORMAL DEVELOPMENT OF THE LEG BONES OF CHICKENS WITH RESPECT TO THEIR ASH CONTENT[1]

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INTRODUCTION

The ash content of certain bones, especially those of the legs, has been used in recent years as a measure of the effect of experimental diets on the mineralization of the skeleton of the animals to which the diets were fed. This has been particularly true in some of the nutrition studies with chickens, in which the percentage of ash in one or another of the leg bones has been used to determine the extent of the occurrence of rickets. The ease with which rickets may be produced in chickens has led to the use of chickens as laboratory animals for determining the antirachitic value of the various vitamin D supplements, particularly cod-liver oil, used in diets for poultry.

In order that a correct interpretation may be placed on the results of work of this nature, it is necessary that data on the ash content of the bones of birds reared under normal environmental conditions be available for the purpose of comparison. Furthermore, these data should furnish information on the effect, if any, of age, breed, and sex on the ash content of the bones. The published percentages of ash which have been considered as normal were obtained almost wholly from the analysis of the bones of birds reared under rather artificial conditions, and they represent only one or, at best, a few ages. It is necessary also to know how the several factors other than diet affect the deposition of the mineral elements in bone, for otherwise one cannot be certain that the results obtained are due only to the diets, or, more specifically, to the vitamin D supplements being studied.

Furthermore, it is often desirable to compare the results of work carried out in different laboratories, and in order that this may be possible it is necessary that comparable methods be used for the determination of the ash in the bones of the experimental birds. At present there does not seem to be any great degree of unanimity among the various investigators in this respect. In general the tibia has been used for this purpose, but in some cases other bones have been used. Some reports show different ways of preparing the bones for analysis; others show the use of different methods for ash determination. Some of these differences probably had little or no effect on the results obtained, but it is also probably true that some had a marked effect.

This lack of uniformity in the methods used for the determination of the ash of the leg bones, as well as the lack of adequate information regarding the percentage of ash in the bones of chickens reared under normal conditions, made it desirable to ascertain the ash content of the bones of such chickens of both sexes and of different ages and breeds by the use of a simple and rapid method.

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PLAN OF THE INVESTIGATION

It was planned to rear two groups of chickens, each group of a different breed, under favorable conditions on a grass range, and to study the normal development of their leg bones with respect to the ash content. Each week, for 20 weeks, representative birds were to be selected from each group to furnish the bones to be studied. The plan involved the determination of the ash content of each of the three major long bones of the right leg and of the combined bones of the left leg (1) with the epiphyseal cartilages retained and (2) with the epiphyseal cartilages removed.

To obtain supplementary information as a check on the ash content of the bones, it was planned to determine the calcium and phosphorus content of the blood serum and of the ash of the tibiae, and to note, by means of X-ray shadowgraphs, the age at which calcification occurred at the distal and proximal ends of the tibiae and the proximal end of the metatarsus. The blood-serum studies were conducted for 25 weeks; the other studies for 20 weeks.

EXPERIMENTAL MATERIAL AND METHODS

Three hundred and fifty chicks of each of the two breeds used (Rhode Island Red and Single-Comb White Leghorn) were hatched on April 8, 1931, at the United States Animal Husbandry Experiment Farm, Beltsville, Md., and were placed, the next day, in colony brooder houses on a grass range. At that time 100 chicks of each breed were selected at random and wing-banded. These birds were weighed then and at intervals of 2 weeks thereafter for 20 weeks, in order to obtain a reliable indication as to whether the average growth of the birds was satisfactory.

For the first 14 weeks, the birds were fed the following dry mash:

	Percent
Yellow corn meal	41. 50
Ground wheat	20. 00
Wheat bran	15. 00
Dried buttermilk	10. 00
Meat scrap (50 percent)	10. 00
Calcite	2. 75
Salt	. 75
Total	100. 00

In addition to this mash, during the first 6 weeks after hatching the birds were given all the freshly soured skim milk they would consume. After the fourteenth week and until the end of the twentieth week, all except 2.5 percent each of the meat scrap and dried buttermilk was replaced by yellow corn meal.

On the day after the chicks were hatched and at intervals of 1 week thereafter, a sufficient number of individuals of each breed was selected at random from among the unbanded birds to furnish the leg bones necessary for the ash determinations. The number for each sample varied from 1 to 15, depending on the size of the chicks.

Calcium and inorganic phosphorus determinations were made on the blood serum of the birds selected, in order that it might be known whether they were normal in this respect. Blood samples of approximately equal size were obtained by heart puncture from each of these birds before they were killed for bone samples. The blood

samples of birds of the same sex and breed were then combined for analysis. It was not possible, however, to differentiate readily between the sexes until the age of 6 weeks in the case of the Leghorns, and until the age of 7 weeks in the case of the Rhode Island Reds. Accordingly, until the sex of the chicks could be determined, the blood samples were combined with regard to breed only. The serum, obtained by centrifuging the blood samples, was analyzed for calcium by the Clark and Collip (*2*) [2] modification of the method of Kramer and Tisdall; and for inorganic phosphorus, by the method of Fiske and Subbarow (*3*).

After the collection of the blood samples, the birds were killed by cutting the jugular vein and puncturing the brain. Immediately after they were killed, X-ray shadowgraphs were made of the legs in order that the calcification of the bones might be studied by this means. The leg bones were then removed and dissected free of flesh in preparation for ashing. The epiphyseal cartilages were removed from the bones of one-half of the birds but not from the bones of the other half. Thus, one-half of the samples were made up of the diaphysis alone, and the other half included the diaphysis and the epiphyses. The long bones (femur, tibia with fibula, and metatarsus) of the left leg were combined in one sample, whereas the femur, tibia without fibula, and metatarsus of the right leg were studied separately.

During the first 6 weeks, the following numbers of birds were used in preparing each sample at the ages indicated.

Number of birds represented in each sample: *Age*
    15_____ 1 day.
    12_____ 1 week.
    8_____ 2 weeks.
    7_____ 3 weeks.
    6_____ 4 weeks.
    6_____ 5 weeks.
    5_____ 6 weeks.

After the age of 6 weeks, one bird was sufficient for the weekly sample.

Bone samples of chicks of each sex were obtained as soon as the males could be readily distinguished from the females. As each bone was freed of flesh and its periosteum, it was placed in 95-percent ethyl alcohol and kept there until the determination of its ash content was begun. The following method of obtaining the ash content of the bones was used: The bones were removed from the alcohol, cut into small pieces with bone snips if they were inconveniently large, wrapped in filter paper, and extracted for 6 hours with 95-percent ethyl alcohol in a Soxhlet extractor. They were next allowed to dry in the open air and then extracted with diethyl ether for 6 hours. After being dried again in the air, the bones were placed in weighed crucibles, dried in a vacuum oven for 4 hours at a temperature of 60° C., and weighed. The weighed, oven-dried samples were then ashed to a constant weight, at 600°, to obtain the weight of ash in the moisture- and fat-free bones.

Previous, but unpublished, work at the United States Animal Husbandry Experiment Farm has shown that this method is efficient from the standpoint of accuracy and rapidity of determination. The

[2] Reference is made by number (italic) to Literature Cited, p. 1008.

extraction should be continued only long enough to insure removal of the soluble material. For this purpose, the writers have found 6 hours with each solvent to be sufficient, for the percentage of ash after such treatment was fully as high as that reported by St. John and his coworkers (9) when much longer periods were employed.

The amount of calcium and phosphorus in the ash of the tibiae was then obtained.[3] For this purpose, the ash was dissolved in dilute hydrochloric acid and the resulting solution was made up to a definite volume. Aliquot parts of this solution were used for the determination of calcium according to Kramer and Howland's (8) method for bone, and for the determination of phosphorus by the gravimetric method of the Association of Official Agricultural Chemists (1) for fertilizers.

EXPERIMENTAL RESULTS

GROWTH OF THE BIRDS

The average growth (as measured by weight in grams) of the birds which were banded is shown in figure 1. There were 35 males and 44 females of the White Leghorn breed and 37 males and 45 females of the Rhode Island Red breed. The curves show that the growth was satisfactory as compared with the growth previously obtained at the Beltsville experiment farm with these breeds (5, 10).

FIGURE 1.—Average weights, at 2-week intervals, of males and females of the two breeds compared with normal weights reported for these breeds by Hendricks, Lee, and Titus (5) and by Titus and Jull (10).

CALCIUM AND INORGANIC PHOSPHORUS CONTENTS OF BLOOD SERUM

The calcium and inorganic phosphorus contents of the blood serum of the birds at intervals of 1 week are shown in figure 2. All the values obtained were well within the normal limits, as shown by unpublished results; in fact, there was less variation than is frequently observed under experimental conditions. The variation was so small throughout most of the first 20 weeks as to have no apparent significance. Shortly after the reduction of the relative quantities of the protein concentrates in the diet of the birds at the age of 14 weeks, there was a decrease for about 4 weeks in the inorganic phosphorus content of the blood serum, whereas the calcium content showed

[3] The determinations of the calcium and phosphorus in the ash of the tibiae were carried out by R. E. Davis and G. H. Kennedy, of the Animal Husbandry Division.

no change. This decrease in phosphorus content was then followed by an increase for 2 or 3 weeks and then by another decrease. There was, apparently, no significant difference in the calcium and inorganic phosphorus contents of the blood serum of the males and females from the sixth or seventh week, when sex was first differentiated, to the twentieth week.

Determinations of the calcium and inorganic phosphorus in the blood serum of the birds were continued from the ages of 20 to 25 weeks, with the special object of observing the changes in the calcium of the blood serum of the pullets as they began to lay, as compared with those changes in the blood serum of the males of the same ages. Figure 2 shows a very marked increase in the case of the White Leghorn pullets after the twentieth week and a similar increase in the case of the Rhode Island Red pullets after the twenty-third week. This increase was undoubtedly due to the increased demand for calcium to be used in egg production, as the pullets began to lay at about this time. Halnan (*4*) has shown that there is an increased retention of calcium from the feed during the week preceding the beginning of egg production, and Hughes and his coworkers (*7*) have found that there is an increased content of calcium in the blood serum of pullets at the time that laying begins.
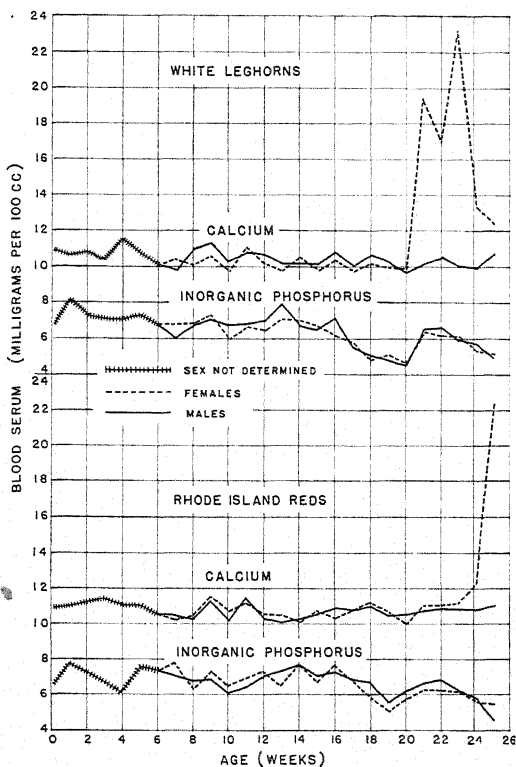
FIGURE 2.—Calcium and inorganic phosphorus contents of the blood serum of White Leghorns and Rhode Island Reds at weekly intervals.

CALCIFICATION OF EPIPHYSES

Through an examination by the X-ray shadowgraphs, certain stages in the calcification of the epiphyses were noted. During the first week of the life of the chick, centers of ossification appeared in the cartilages at the distal end of the tibia and the proximal end of the metatarsus. There was no difference between the two breeds in this respect. Centers of ossification appeared in the cartilage at the proximal end of the tibia by the end of the sixth week. In this respect also no difference was noted between the two breeds.

After the first appearance of the centers of ossification the areas undergoing calcification gradually increased in size until they made up most of the epiphyses and the epiphyses became attached to the

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diaphysis. The union of a diaphysis with an epiphysis occurred first at the proximal end of the metatarsus, then at the distal end of the tibia, and later at the proximal end of the tibia. There seemed to be a tendency for the epiphyses of the females to calcify at an earlier age than those of the males, and for those of the White Leghorn birds to calcify at an earlier age than those of the Rhode Island Reds (table 1).

TABLE 1.—*Effect of breed and sex on the age at which the calcification of the epiph-yses were apparently complete, as shown by the X-ray shadowgraphs*

Breed and sex of birds	Age of bird when calcification occurred at —		
	Proximal end of meta-tarsus	Distal end of tibia	Proximal end of tibia
White Leghorn:	*Weeks*	*Weeks*	*Weeks*
Female	12	13	19
Male	14	15	20
Rhode Island Red:			
Female	13	17	20
Male	14	19	(a)

a Not complete at twentieth week.

ASH CONTENT OF BONES

The percentage of ash in the moisture- and fat-free bones each week of the experiment is presented in figures 3 and 4, and the percentages
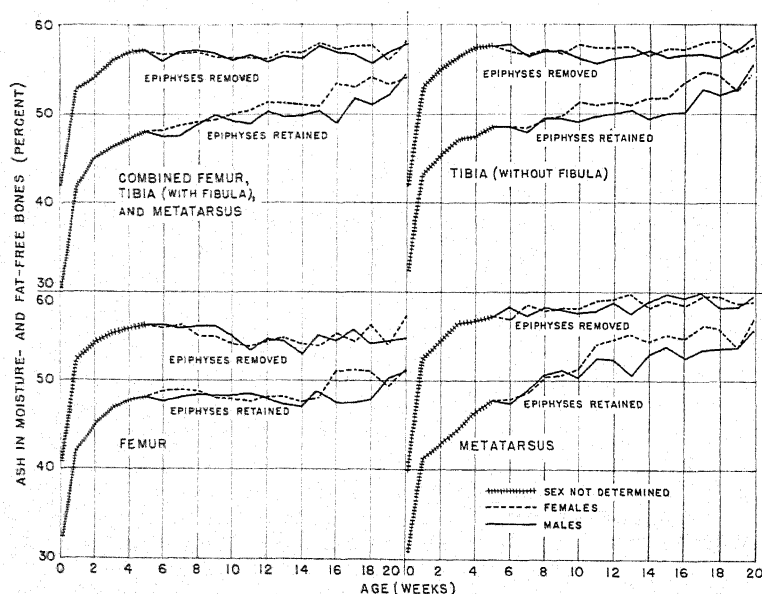
FIGURE 3.—Ash content of the bone samples obtained from the White Leghorns at weekly intervals.

at 4, 5, 6, 7, 8, and 20 weeks are given in table 2. The curves are com-paratively smooth during the first few weeks, when the determina-tions of ash were made on samples composed of bones of from 5 to 15 individuals. Later, when the samples represented single birds, these curves are not so smooth.
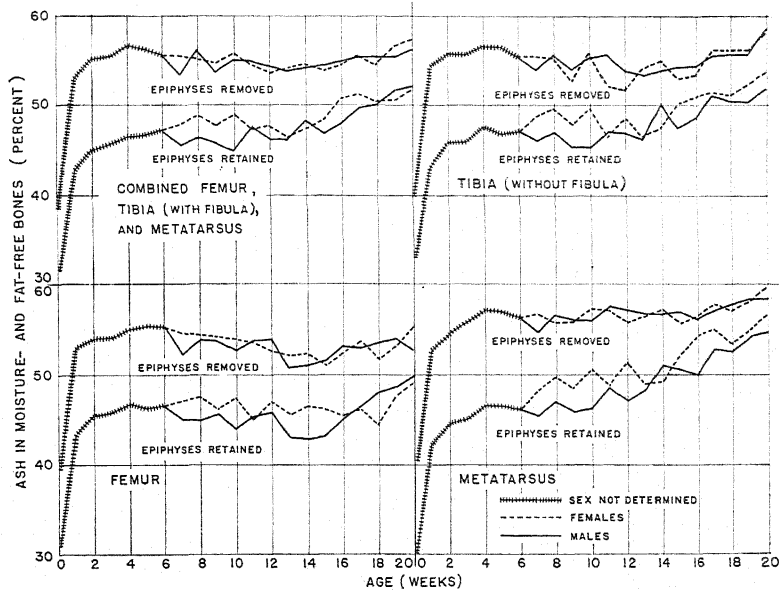
FIGURE 4.—Ash content of the bone samples obtained from the Rhode Island Reds at weekly intervals

TABLE 2.—*Percentage of ash in the moisture- and fat-free bones of the White Leghorns and the Rhode Island Reds at the ages indicated*

WHITE LEGHORNS

Age in weeks	Sex	Bones with cartilage retained				Bones with cartilage removed			
		Femur	Tibia	Meta-tarsus	Com-bined bones	Femur	Tibia	Meta-tarsus	Com-bined bones
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
4	Not separated	47.8	47.5	46.4	47.3	55.9	57.4	56.7	56.9
5	....do	48.2	48.4	47.7	48.1	56.1	57.6	57.2	57.1
6	Male	47.8	48.7	47.6	47.6	56.3	57.8	58.4	56.0
6	Female	48.8	48.6	47.6	48.1	56.0	57.1	56.8	56.9
7	Male	48.2	48.1	49.0	47.6	56.0	56.4	57.3	57.1
7	Female	48.8	48.4	48.6	48.8	56.2	56.7	58.4	56.8
8	Male	48.5	49.5	50.8	49.0	56.0	57.0	58.3	57.2
8	Female	48.7	49.4	50.5	49.0	55.1	57.3	57.9	56.9
20	Male	50.9	55.8	55.9	54.4	54.8	59.6	59.4	57.9
20	Female	51.2	54.6	56.8	54.3	57.3	57.7	58.8	58.2

RHODE ISLAND REDS

Age in weeks	Sex	Bones with cartilage retained				Bones with cartilage removed			
4	Not separated	46.7	47.6	46.5	46.5	55.0	56.6	57.0	56.7
5	....do	46.3	46.8	46.5	46.6	55.3	56.4	56.9	56.5
6	....do	46.4	47.0	46.1	47.3	55.3	55.4	56.3	55.6
7	Male	45.0	46.1	45.5	45.9	52.2	54.0	54.6	53.5
7	Female	47.1	49.0	48.3	48.0	54.6	55.4	56.8	55.5
8	Male	45.0	47.0	46.9	46.6	53.7	55.8	56.6	56.1
8	Female	47.4	49.8	49.7	49.1	54.4	55.4	55.8	55.1
20	Male	50.0	51.8	54.4	52.2	52.6	58.5	58.3	56.0
20	Female	49.0	53.6	56.6	51.8	55.3	58.5	59.8	57.4

In general, the ash content of the moisture- and fat-free bones of the chicks at the age of 1 day was approximately 40 percent when the epiphyseal cartilages were removed and 30 percent when they were

retained. There was a marked increase in the ash content during the first week, and this increase continued, but at a progressively slower rate, during the next few weeks. Thereafter, there was relatively little change.

A comparison of the curves representing the ash content of the bones, with and without the epiphyseal cartilages, shows that the diaphysis contains an appreciably higher percentage of ash than does the diaphysis with the epiphyses. This difference is greatest during the first few weeks, after which the curves representing the percentage of ash in the bones prepared in the two different ways tend to approach each other gradually and are fairly close together at the end of 20 weeks.

The ash content of the bones obtained from the White Leghorns was higher than that of the corresponding bones of the Rhode Island Reds. This difference continued throughout most of the period of 20 weeks covered in the present study. After approximately the nineteenth week, however, there was a tendency for the ash content of the bones of the Rhode Island Reds to become very nearly equal to that of the bones of the White Leghorns. Thus it appears that calcification of the leg bones proceeds at a higher rate and is completed at an earlier age in the White Leghorns than in the Rhode Island Reds. Such a difference is to be expected, since the former matures somewhat more rapidly than the latter.

Another point brought out by these curves is that throughout most of the period when the males and females were studied separately the average ash content of the leg bones of the females from which the cartilage had not been removed was higher than that of the leg bones of the males. However, at the twentieth week there was, in general, very little difference. In contrast to this, the ash content of the bones from which the cartilage had been removed was practically the same for the two sexes.

A comparison of the three long bones of the leg shows that the metatarsus had the most ash, the femur the least, and the tibia an intermediate amount. During the preparation of the samples a corresponding difference was noted in the physical character of these three bones. The metatarsus was yellowish white in color, had a yellow marrow, greater strength, and less brittleness than either of the other two bones. There was less difference between the femur and the tibia. Both of them were grayish white, had a red marrow, and tended to shatter readily when cut with the bone snips, in contrast to the metatarsus which permitted the bone snips to cut through cleanly. The femur, however, seemed to be somewhat more brittle than the tibia. Thus, it appears that the ash content of the bones bears some relation to their physical character.

CALCIUM AND PHOSPHORUS CONTENTS OF THE ASH OF THE TIBIA

There was relatively little change in the calcium and phosphorus contents of the ash of the tibiae throughout the 20 weeks and, for this reason, only the averages of the results obtained are given in table 3. After the first week the values ranged between approximately 34.5 and 37 percent of calcium and 17.5 and 18.5 percent of phosphorus. There was apparently no difference in this respect between the bones of cockerels and pullets or between birds of different ages.

TABLE 3.—*Effect of age and sex on average percentage of calcium and phosphorus in the ash of the tibiae of White Leghorns and Rhode Island Reds*

WHITE LEGHORNS

Age of birds	Sex of birds	Bones with cartilage retained			Bones with cartilage removed		
		Ca	P	Ca/P ratio	Ca	P	Ca/P ratio
		Percent	*Percent*		*Percent*	*Percent*	
1 day	Not separated	31.3	17.7	1.77:1	35.0	20.4	1.72:1
1-5 weeks	do	35.0	18.1	1.94:1	34.2	17.8	1.94:1
6-20 weeks	Male	35.7	17.9	1.98:1	36.7	18.2	2.02:1
	Female	35.9	17.6	2.05:1	36.2	18.1	2.00:1

RHODE ISLAND REDS

1 day	Not separated	33.4	18.9	1.77:1	35.0	19.8	1.77:1
1-6 weeks	do	34.5	17.9	1.93:1	34.8	17.9	1.96:1
7-20 weeks	Male	35.3	17.8	1.97:1	36.1	18.1	1.99:1
	Female	36.7	18.4	2.00:1	36.7	18.4	2.00:1

The ratio of calcium to phosphorus in the ash of the tibiae was found to be very close to 2.0:1 in most of the samples; therefore the averages of the values, given in table 3 for the different breeds and sexes, in most instances are also very close to 2.0:1. However, one exception to the foregoing statement is that of the calcium-phosphorus ratio of the ash of the tibiae in birds aged 1 day. The average ratio was 1.77:1 in three cases and 1.72:1 in the fourth.

DISCUSSION

The data presented in this paper on the ash content of the moisture- and fat-free leg bones of chickens indicate that there was little tendency for the percentage of ash in those bones from which the cartilage had been removed to change after the fourth or fifth week. There appears, however, to have been a slight tendency for the ash content of the metatarsus to increase with the age of the chick and for that of the femur to decrease between the fifth and fifteenth week. Nevertheless, in both cases the maximum deviation from the average ash content observed between the seventh and twentieth week was less than 4 percent of the average.

Although the data show that the ash content of the bones from which the epiphyseal cartilages had been removed tended to remain rather constant from the fourth to the twentieth week, they also show that there was a gradual increase, during this period, in the ash content of the bones (other than the femur) from which the cartilages had not been removed. It appears, therefore, that this change was due to a progressive calcification of the epiphyseal cartilages. In any case, the ash content of the bones from which the cartilages had not been removed tended to become more nearly equal to that of the bones from which the cartilages had been removed as the birds approached maturity and the calcification of the epiphyses became more nearly complete.

The tendency for the ash content of the diaphysis to remain rather constant under normal conditions does not necessarily mean that the ash content may not change under certain conditions, for unpublished

experiments by the writers have shown that it may change either as the result of the occurrence of rickets or because of a change in the rate of growth. If the rate of growth is slow, the cartilage-free bones may have an abnormally high ash content. On the other hand, very rapid growth, especially after a period of slow growth, tends to bring about a decrease in the ash content of both the diaphysis and the epiphyseal cartilage. It is quite probable that much of the variation among individual chickens of the ash content of their leg bones is due largely to differences in their rate of growth.

It is necessary, therefore, to consider the rate of growth of the chickens in any experimental or regulatory work with feeds in order that errors in the interpretation of results may not occur. Thus it is possible that a diet containing a cod-liver oil low in vitamin A might contain an inadequate supply of this vitamin. The chickens receiving such a diet would tend to grow more slowly than others receiving an adequate supply, and probably the percentage of ash in their bones would be higher than if their growth had been normal. Failure to take the growth rate into account would lead to the conclusion that the antirachitic value of the oil under consideration was higher than was actually the case.

The results of the present study indicate that, in most cases, when the birds are between the ages of 7 and 20 weeks, the ash content of the females' bones with the cartilage retained is greater than that of the males' bones. The X-ray shadowgraphs furnish evidence that this difference is due to an earlier calcification of the epiphyses of the bones of the females rather than to a greater storage of minerals in preparation for egg production, as was suggested by Holmes, Pigott, and Moore (6). Furthermore, the bones of the males, either with or without their cartilages, contain approximately the same proportion of ash at the age of 20 weeks as do those of the females. Moreover the greater weight of the bones of the males, as is evident from their greater body weight, indicates a larger total quantity of ash stored in their skeletons.

Since the above-mentioned difference was found to be due to an earlier calcification of the cartilages in the case of the females, it can be avoided in experimental studies by removing the epiphyseal cartilages from all bones to be ashed. Furthermore, if the epiphyseal cartilages are removed in preparing the samples, the writers' results indicate that it is unnecessary to consider the sex of the individual or its age, between the fifth or sixth and the twentieth week. The removal of the cartilages in the preparation of the bone for ashing makes the dissection of the bone easier and more accurate, for the cartilages can be removed very readily with the flesh and periosteum. The accuracy of the dissection is an important point, for the writers have found, in unpublished work, that there is more danger of error resulting from faulty dissection than from any other step in the analytical technic.

The objection may be raised that the diaphysis alone would not indicate the extent of the occurrence of rickets so well as would the diaphysis together with the epiphyseal cartilages. St. John, Kempf, and Bond (9) found that between normal and rachitic chicks there was a greater difference in the ash content of the epiphyseal cartilages than in that of the diaphyses. Although this is true, it is also true

that after the first few weeks the epiphyses make up only a small portion of the combined weight of the bone and cartilages and would, therefore, have less influence on the ash content of the sample than would the diaphysis. The writers have found, in unpublished data, that the ash content of the diaphyses of bones obtained from rachitic chicks is almost as low as the ash content of the diaphysis and epiphyses combined. They conclude, therefore, that it is entirely satisfactory to use the percentage of ash in the diaphyses of the leg bones as a measure of the extent of the development of rickets in the chicken.

The data obtained by the writers seem to indicate that the tibia would ordinarily be most satisfactory for determining the ash content of the leg bones of chickens reared under normal conditions. The ash content of the tibia agrees closely with that of the combined long bones of the leg, and the use of the tibia alone has the advantage of simplifying the analytical procedure. This bone has also been generally used in the work reported in the literature.

SUMMARY AND CONCLUSIONS

Two groups of chickens, one consisting of 350 Single-Comb White Leghorns and the other of 350 Rhode Island Reds, were reared on a grass range under normal conditions. The ash content of the moisture- and fat-free leg bones of representative individuals from each group was determined each week during the first 20 weeks of their lives. Stages of calcification of the epiphyses in the leg bones of these birds were noted by means of X-ray shadowgraphs. The contents of the calcium and inorganic phosphorus of the blood serum and of the ash of the tibiae also were obtained.

There was no significant change in the calcium content of the blood serum of the birds with increasing age, except for a marked increase in the case of the pullets just before they commenced to lay. After the fourteenth week there were marked changes in the inorganic phosphorus content of the blood serum, which were probably due in part to increasing age and in part to a change in the diet which was made at that time.

The X-ray shadowgraphs indicated an earlier calcification of the epiphyses of the bones of the females than of the males and also an earlier calcification of the epiphyses of the bones of the White Leghorns than of the Rhode Island Reds.

There were no significant differences in the calcium and phosphorus contents of the ash of the tibiae, nor was the difference significant between the sexes. The ratio of calcium to phosphorus in the ash of the tibiae was noticeably low on the day after hatching, after which it increased to approximately 2.0:1 in all cases.

The ash content of the diaphysis of the long bones of the legs of chickens of both sexes tended to remain constant between the fifth and twentieth weeks. In general, the ash content of the diaphysis with its cartilages was higher in the case of the females than in the case of the males between the sixth or seventh and the eighteenth week, depending on the breed.

It is concluded that the tibia is the most satisfactory bone to use in studying the effect of various diets and sources of vitamin D on the ash content of the bones of chickens. It is recommended that the epiphyseal cartilages, together with the periosteum, be removed in the preparation of the bone for ashing.

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THE LETHAL EFFECT OF LOW TEMPERATURES ON THE VARIOUS STAGES OF THE CONFUSED FLOUR BEETLE [1]

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INTRODUCTION

Entomologists know that stored-product insects are not very resistant to low temperatures. Refrigeration is employed commercially to prevent insect damage. Ordinarily, insect activity is suspended in infested articles, or the infestation by insects is prevented in "sterilized" goods. However, low temperature is rarely used as the sterilizing agent in place of heat or a fumigant.

As compared with the attention paid to the effect of high temperature, but little investigation has been made of the lethal effect of low temperatures. It must be recognized that the cold resistance of different species of insects may vary widely, whereas the heat resistance varies only slightly. In order to obtain the most economical combination of the temperature and the length of exposure, actual data are necessary regarding the time required to kill a certain insect species at any given low temperatures.

Because the confused flour beetle (*Tribolium confusum* Duval) is an insect with which prepared food products are likely to become infested, it was selected for the study reported in this paper. The data presented are of particular interest also for their physiological significance.

In applying time-temperature data to the effect of low temperatures on insects, it should be understood that allowance must be made for the time necessary to cool the insect-containing medium, as, for example, wheat flour. The measurement of the cooling (heat transfer) time of standard volumes of various commercial products is a separate study which will not be considered here.

HISTORICAL REVIEW

Most of the data available in the literature regarding the lethal effects of low temperatures on stored-product insects are merely isolated observations, often presented without the time of exposure being reported. The first accurate measurement of the effect of refrigeration on an insect was made by Back and Pemberton (*1, 2*),[2] who found it possible to kill the early stages of the Mediterranean fruit fly by cold storage.

Although some data published relative to this subject are available for other stored-product insects, only one reference to the effect of low temperatures on *Tribolium confusum* is known. Payne (*8*) says that normally it may recover from temperatures as low as $-14°$ C., but she makes no mention of the duration of the exposure.

EXPERIMENTAL METHOD

A Carrier air-conditioned cabinet was employed in the experiments involving temperatures from 0° to −18° C. A variation of less than 1° was obtained for the period of each experiment. For the experiments in which higher temperatures were used another type of cabinet was utilized in which the temperature varied only slightly over a period of several weeks.

Tribolium confusum were cultured in fine whole-wheat flour, about 500 grams for each lot. The flour was sifted through a standard no. 6 silk bolting cloth (74 meshes to the linear inch). About 3,000 adult beetles were placed in each lot. The cultures were covered with muslin and kept at a constant temperature of 27° C. Eggs were seldom allowed to accumulate more than 24 hours.

All stages except the adults were exposed in small glass vials, 50 individuals in each, with a few exceptions when 100 were so exposed. The eggs were counted under a binocular microscope, only those which were normally plump being used. The unstoppered vials with their contents were placed in the temperature cabinets. After exposure each vial was lightly stoppered with cotton or with cork stoppers having screen-covered holes. Adults were exposed in silk bolting-cloth cylinders. This precaution was necessary because preliminary tests had shown that the excited adults give off an odoriferous vapor. When confined with this gas they soon die, 95 percent being killed in 4 or 5 hours.

During the earlier experiments exposed eggs without flour were placed in a cabinet and kept at a temperature of 27° C. During the hatching, results were noted daily, the young larvae being removed from the vials and discarded. Later tests showed that the results were the same if eggs were allowed to incubate in about 2 grams of flour and the larvae counted 3 or 4 days after hatching began. The tedious work of noting results was thus greatly reduced. All other stages of the insect were treated in this manner. Check experiments of 50 individuals each were always included in each series. They were kept at 27° and their condition noted only after the observations on the exposed insects had been completed.

All experiments at 7° C. were carried out in glass desiccators at three different relative humidities, viz. 6.2 (7-mm saturation deficiency), 50, and 73.7 percent. With the exception of five experiments which were made without moisture control, all experiments at 12° were carried out likewise in desiccators at relative humidities of 32.8 (7-mm saturation deficiency), 50, and 73.7 percent. At both temperatures no differences in the lethal effects at different humidities were observed, neither did there appear to be any difference in the results obtained from the experiments conducted outside the desiccators. Accordingly, all the experiments at each of these temperatures were grouped without regard to moisture conditions.

The percentage of mortality in each case was calculated by Abbott's formula, $\frac{x-y}{x}$ (100), where x is the percentage survival in the check and y that in the exposed lot. In figure 1 are shown curves for eggs of three age groups at 12° C. Fifty and 100 percent mortalities were estimated in all cases from curves of this type. Each point in the curve shown is an average of the results of from 1 to 14 experiments,

each involving 50 individual insects. Although a few points are based on a single experiment, no curve is drawn through less than eight points.

INFLUENCE OF AGE ON COLD RESISTANCE OF EGGS

Davis (7), studying the effects of high and low temperatures upon the mosquito *Aedes aegypti* Linn., found that at $-5.5°$ C., freshly laid eggs were slightly more resistant than mature eggs. The writers have found the same thing to be true for eggs of *Tribolium confusum* that have been exposed to chloropicrin.

In order to study the effect of age on the resistance of eggs of *Tribolium confusum* to low temperature, eggs were placed in six age groups, namely, 1 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, and 120 to 132 hours old. At 27° C., the temperature at which the sifted eggs were stored, those 132 hours old were nearly ready to hatch. Eggs in the different age groups were simultaneously exposed to low
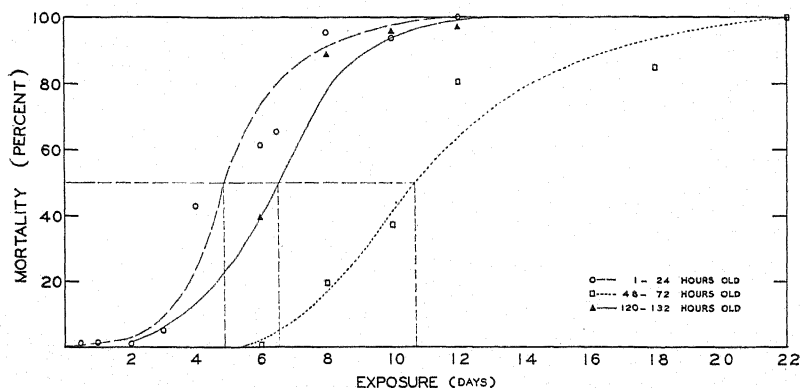
FIGURE 1.—Mortality of eggs of *Tribolium confusum* of different ages when exposed to a temperature of 12° C.

temperatures and otherwise treated as nearly alike as possible. In counting the survivors of these tests, only those larvae which previous experience showed to be able to complete their development were counted. Certain larvae died after emerging partly from the egg. Others hatched in such a weakened condition that they also soon died. All such larvae were considered as having been killed in the egg stage. The eggs set aside as checks showed from 78.38 to 90 percent survival as calculated from the number of young larvae hatched from them.

Figure 1 shows the average results at each age-temperature combination. In table 1 is given a summary of the time required at the various temperatures to obtain 50 and 100 percent mortality. The latter figures do not possess, for comparative purposes, the precision of the previous ones, but they are more useful in estimating time-temperature relations for insect control. Although eggs were separated into 6 age groups most of the experiments were confined to 3 ages, namely, 1 to 24, 48 to 72, and 120 to 132 hours old. A few results with eggs at other ages are given for the sake of showing the influence of the entire range of age on the lethal effects of temperature at $-12°$ C.

TABLE 1.—*Effect of low temperatures on eggs of Tribolium confusum of different ages, 50 and 100 percent mortality being the basis of comparison*

50 PERCENT MORTALITY

Temperature (° C.)	Number of hours of exposure required to produce mortality in eggs of—					
	Age 1 to 24 hours	Age 24 to 48 hours	Age 48 to 72 hours	Age 72 to 96 hours	Age 96 to 120 hours	Age 120 to 132 hours
12	118		258			157
7	43		214			110
0	4.75	4.5		11.25		
−4	1.5	1.5	3.5	3.75	2.75	
−6	.75		3.25			4
−12	.75	1.5	3.5	6	2.75	1.5
−12 [a]	.75		.75			1.25
−18	.25		.25			.25

100 PERCENT MORTALITY

Temperature (° C.)	Age 1 to 24 hours	Age 24 to 48 hours	Age 48 to 72 hours	Age 72 to 96 hours	Age 96 to 120 hours	Age 120 to 132 hours
12	288		528			312
7	216		432			288
0	13	11.5		16	13.5	
−4	9.25	8.25	7.5	9.5	8.25	
−6	6.5		8			13
−12	9.5	9.5	14	12	9.5	6
−18	1		1			1

[a] The second series of experiments at −12° C. included fewer eggs for the determinations at 1 to 24 hours and at 48 to 72 hours than in the first series at that temperature.

The results show that in general the resistance of the eggs is probably greatest in the fourth age group which includes eggs 72 to 96 hours old. According to available embryological data for *Tribolium confusum* at this age, the embryos have just undergone dorsal closure and the young larvae are becoming rather well formed. It is known for insect embryos in general that their respiration is at a lower level here than at other stages of their development.

INFLUENCE OF AGE ON COLD RESISTANCE OF LARVAE

For the purposes of the experiments with larvae of *Tribolium confusum* three age groups were selected. The first group consisted of unfed larvae 1 to 12 hours old, averaging 6 hours. The second and third groups of about half-grown and of full-grown larvae, respectively, were raised on whole-wheat flour, from eggs the age of which was known for each lot. Because larvae of the same age varied in size considerably under the culture conditions, a few third- and sixth-instar larvae were selected by the aid of the measurements given by Chapman (5) and by Brindley (3). Then by comparison it was possible to select with a fair degree of accuracy the larvae of these instars from the cultures in which they predominated. Only active larvae able to resist jarring from a sheet of paper were used, thus eliminating those in the process of molting.

The manner in which the larvae were handled was practically the same as that for the eggs. Fifty individuals were used in each experiment. Observations were made regularly 1 week after treatment. The groups of larvae set aside as checks showed 91.2 to 100 percent survival.

In table 2 is summarized the time in hours required to obtain 50 and 100 percent mortality for the three age groups of larvae at different low temperatures. These data indicate that the full-grown larvae are most resistant, although the difference is pronounced only at 7° C.

Larvae killed outright by freezing invariably turn black soon afterward. Some others turn black in irregular areas. Many larvae which otherwise appeared normal were unable to void feces properly. In some cases long, sticklike strands protruded from the anus a distance equivalent to the length of the entire body. In full-grown larvae certain time-temperature combinations produced rare winged freaks, examples of metathetely.[3]

TABLE 2.—*Effect of low temperatures on larvae of Tribolium confusum at different stages of growth, 50 and 100 percent mortality being the basis of comparison*

50 PERCENT MORTALITY

Temperature (° C.)	Number of hours of exposure required to produce mortality in larvae of—		
	Age 1 to 12 hours	Third instar	Sixth instar
7	134.4	149	197
−6	10	9	11.75
−12	.42	.35	.48
−18	.18	.17	.17

100 PERCENT MORTALITY

7	288	250	528
−6	16	16	16
−12	1.75	.5	1
−18	1	.5	.5

EFFECT OF LOW TEMPERATURES ON PUPAE

Pupae of only one age were selected. In general, the lighter-colored ones are youngest, and these were chosen for use in the tests. Each lot of treated pupae was placed in about 2 grams of flour at 27° C. Mortality was based on the percentage emergence of the adults. The groups of pupae set aside as checks showed a high proportion of emergence, 96.32 to 100 percent.

In table 3 are summarized the times in hours required to obtain 50 and 100 percent mortality of pupae at different low temperatures. Results at 12° C. are not tabulated because exposures over a long time showed very little mortality. The keeping of 100 pupae at 12° for 22 days resulted in only 4 dead specimens. No adults emerged during this long exposure.

TABLE 3.—*Effect of low temperatures on pupae of Tribolium confusum*

Tempera-ture (° C.)	Hours of exposure required to produce—	
	50 percent mortality	100 percent mortality
7	258	432
− 6	.67	10
−12	.63	1.5
−18	.13	.5

[3] Chapman (*6, p. 294*) refers to Strickland's study of parasitized *Simulium* larvae in which Strickland found that the action of the parasite prevented the wing histoblasts from developing normally, the wing pads being greatly retarded and reduced. To this phenomenon, Strickland gave the term "metathetely" (to run behind completion).

EFFECT OF LOW TEMPERATURES ON ADULTS

Adults ranging in age from 1 to 5 months were used, no attempt being made to study the effect of age on their resistance to low temperatures. As the adults live a year or more, those used were young and vigorous. Treated adults were kept under observation for a week or so without flour. Check groups of adults under these conditions showed 94.66 to 96 percent survival.

In table 4 are summarized the times in hours required to obtain 50 and 100 percent mortality of adults at different low temperatures. Exposures of adults at 12° C., as in the case of pupae at this temperature, are not tabulated. Some activity and little mortality of adults occurs at this temperature, groups being kept at different humidities for from 22 to 35 days without showing over 6 percent mortality.

TABLE 4.—*Effect of low temperatures on adults of Tribolium confusum*

Tempera- (° C.)	Hours of exposure required to produce—	
	50 percent mortality	100 percent mortality
7	336	528
− 6	8.4	24
−12	.23	2
−18	.15	.5

As mentioned before, it was necessary to place the adults for treatment in bolting-cloth cylinders to prevent accumulation of the toxic vapors given off by the excited beetles. Adults injured by low temperature also give off this vapor, which, if the beetles are placed in flour, imparts a definitely pink color to that flour. Several series of experiments indicated that the intensity of this color varied directly with the length of exposure to low temperature. Chapman (6) noted this color in flour and other materials, stating that the vapor is given off by beetles irritated mechanically.

Adults not injured sufficiently to die as a result of their cold treatment were counted as survivors. It was found that survivors could usually be easily determined after a week was allowed for recovery. In two instances adults exposed to −12° C. for 45 minutes were still alive after 45 days, but they were never able to crawl about. Inability to void feces properly was common among injured adults as in the case of the larvae. Whenever the recovery of an individual was in doubt, it was counted as a survivor. Some living adults, although more or less paralyzed after they had been exposed to −12° for 45 minutes, produced fertile eggs. Survivors of 6 hours exposure to −6° produced many fertile eggs. Four hundred adults were kept in 300 grams of flour at 12° for 29 days, but no eggs were laid at that temperature. Several days after the removal of these adults from the low temperature 400 to 500 eggs were recovered, indicating that egg laying was normal after such a period of inactivity.

RELATIVE RESISTANCE OF DIFFERENT STAGES

Table 5 shows the time-temperature dosages required to kill both 50 and 100 percent of the different stages of the confused flour beetle. In the case of the eggs and the larvae, several ages of which were exposed to low temperatures, the results for the most resistant ages are given.

TABLE 5.—*Effect of low temperatures on the various stages of Tribolium confusum 50 and 100 percent mortality being the basis of comparison*

50 PERCENT MORTALITY

Stage	Number of hours of exposure required to produce mortality at temperature of—				
	7° C.	0° C.	−6° C.	−12° C.	−18° C.
Egg	214	11.25	4	3.5	0.25
Larva	197		11.75	.48	.18
Pupa	258		.67	.63	.13
Adult	336		8.4	.23	.15

100 PERCENT MORTALITY

Egg	432	16	14	14	1
Larva	528		16	1.75	1
Pupa	432		10	1.5	.5
Adult	528		24	2	.5

It is apparent from the figures for 50 percent mortality that the adults are more resistant than the other stages at the moderately low temperature of 7° C. At −6°, however, the larvae are about as hard to kill as the adults and at −12° the eggs are by far the most resistant. The pupae succumb more readily than the other stages.

The figures for 100 percent mortality provide answers to the practical questions of how low a temperature and how long an exposure is necessary to kill all stages of the confused flour beetle. It is possible to kill every stage of the insect at 7° C. (44.6° F.) if held at that temperature for nearly 25 days (600 hours). Only 24 hours' exposure is necessary if the temperature can be lowered to a little below −6° C. (21.2° F.).

DISCUSSION

In no previous instance apparently have the effects of low temperatures on insects been compared on the basis of 50 percent mortality. Carter (*4*) compared the resistance of various stages of the bean weevil (*Mylabris obtectus* Say) on the basis of 100-percent kill. All stages were killed only after several hours exposure to temperatures of −12° to −15° C., whereas *Tribolium confusum* in every stage but the egg succumbs in 2 hours or less at −12°. Robinson (*9*) determined the resistance of the rice and the granary weevils (*Sitophilus oryzae* L. and *S. granarius* L.) to various low temperatures at intervals from their respective thresholds of activity to as low as −17.7°. From a comparison of his data with those given here for *T. confusum* it appears that the latter species is nearly as susceptible to low temperatures as the rice weevil. Given the same low temperature in their environments, it is much easier to kill the confused flour beetle with cold than it is the granary weevil. Robinson reports that *S. granarius* requires 100 hours at −6.6° for a complete kill, whereas it has been shown here that all stages of *T. confusum* are killed at −6° in 24 hours.

SUMMARY

A study was made of the lethal effect of low temperatures on four stages of the confused flour beetle (*Tribolium confusum* Duval)—egg, larval, pupal, and adult. Data were obtained showing the number of

hours of exposure required to produce 50- and 100-percent mortality, the temperatures ranging from 12° to − 18° C.

The eggs were placed in six groups according to age, 1 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, and 120 to 132 hours. Resistance to cold was found to be greatest in the fourth age group, 72 to 96 hours.

The larvae were placed in three age groups—unfed larvae 1 to 12 hours old, half-grown, and full-grown larvae. The full-grown larvae were found to be the most resistant, although the difference was pronounced only at 7° C.

The mortality of the pupae was based on the percentage of emergence of the adults.

The adults ranged in age from 1 to 5 months, and those not injured sufficiently to die as a result of exposure to low temperatures were counted as survivors.

The figures for 50-percent mortality show that the adults were more resistant than the other stages at 7° C.; at − 6° the larvae were about as resistant as the adults; and at − 12° the eggs were the most resistant.

The figures for 100-percent mortality show that all stages of the insect can be killed at 7° C. if exposed at that temperature for 25 days; but if the temperature is lowered to − 6°, only 24 hours' exposure is necessary.

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INCREASE OF KERNEL WEIGHT IN COMMON WHEAT DUE TO BLACK-POINT DISEASE [1]

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INTRODUCTION

This paper deals mainly with the relative weights of kernels of wheat (*Triticum vulgare* Vill.) showing the presence of "black point" and those free from this infection. The main study was made upon kernels from individual wheat plants from the F_5 population resulting from the cross Reward × ((Kota × Marquis) × Hope). The wheat, grown at Fargo in 1933, was planted on May 5, which was about 2 weeks later than the earliest date available for planting in the nursery where it was grown.

A sample of the infected kernels was submitted to H. B. Humphrey, of the Bureau of Plant Industry, United States Department of Agriculture. Dr. Humphrey reported that about one half of the kernels were infected with a strain of *Alternaria*, while the remainder were clearly and positively infected with *Helminthosporium sativum* Pam., King, and Bak. Black point was much in evidence in a number of common wheats in 1933 in the nursery, the percentage of infection varying decidedly with the breeding of the wheat and also, evidently, with the time of planting.

In previous reports upon black point, durum wheats have been held to be most commonly infected (Evans [2] and Weniger)[3] although the infection has not been at all rare upon common wheats. Weniger reports that *Helminthosporium* may produce different types of infection. She mentions head blighting characterized by typical glume lesions and empty spikelets, the latter often occurring several in a group, or as individual empty spikelets (or florets) in any portion of the head. In addition, the black-point type of infection is mentioned as occurring on the seeds. Christensen [4] shows that *H. sativum* attacks different parts of the wheat plant, producing various types of lesions.

According to Christensen the various *Helminthosporium* lesions in wheat or other plants result from repeated inoculations, and Stakman holds that the disease is not systemic.[5] The lesions of black point must be induced then by local spore infection in the ovules. Evidently the time of infection, with regard to the stage of the ovule, and the circumstances attending the development of the fungus until the ripening of the seed, have not been closely studied. Henry [6]

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has shown that high temperatures are favorable for the development of *Helminthosporium*, and moisture conditions are of course important.

EXPERIMENTAL WORK

While taking notes on samples of grain threshed from individual plants, the writer was struck by the apparent differences in the size of kernels from any one plant, depending on whether the kernels showed the presence of black point or whether they were free from the infection. Certain of the plants had the weights in grams per 1,000 kernels shown in table 1.

TABLE 1.—*Weight of healthy wheat kernels and of kernels diseased with black point*

Plant no.	Weight per thousand kernels—	
	Healthy	Black-point
	Grams	*Grams*
48.12.247	27. 1	33. 1
48.12.257	30. 7	35. 9
48.12.259	26. 2	33. 9
48.12.288	30. 2	36. 1
48.12.298	32. 2	38. 4

The infected kernels in these instances weighed about 20 percent more than the kernels showing no infection.

Of the plants in the group studied in detail, about one half were eliminated in the field because of sterile florets. The remaining plants showed a small amount of floret sterility, but with the exception of 1 or 2 spikes, perhaps no more than might be expected in view of the extreme heat which had prevailed.

Sixteen plants were used in the study of relative weights of kernels. The kernels were carefully removed from the spike and laid in order in depressions cut in paraffin blocks and then weighed to fifths of milligrams. The 16 plants had 63 spikes and 2,030 kernels.

A cursory examination suggested that the differences in kernel weight might be ascribed to a differential incidence of black point on the different kernels of the spikelet relative to position. While the third kernel of the spikelet, when present, showed lesions less commonly than the basal kernels, it became evident that this did not entirely explain the differences.

TABLE 2.—*Number and weight of black-point and healthy kernels found paired in the spikelet, unpaired basal kernels, and kernels of the third floret*

Item	Black-point kernels		Healthy kernels		Excess weight of diseased over healthy kernels
	Number	Average weight	Number	Average weight	
		Milligrams		*Milligrams*	*Milligrams*
Paired	344	39. 0±0. 17	1,036	36. 3±0. 13	2. 7±0. 21
Unpaired	34	35. 0± .71	191	32. 5± .37	2. 5± .80
Third floret	19	31. 0± .63	406	27. 1± .20	3. 9± .66
Total or average	397	38. 3± .18	1, 633	33. 5± .12	[a] 4. 7± .22

[a] This value of 4.7 is simply the difference of the weighted means in columns 3 and 5.

The kernels, both those infected with black point and the healthy ones, were placed in three categories. Kernels found paired in the two basal florets comprised the major group; kernels of the third floret, the second group; and unpaired kernels of the basal florets, the third group. The number of kernels, and the average weight per kernel in each of the three classes, and the total weight, are given in table 2.

The elimination of the kernels found in the unpaired and in the third florets reduces the average difference in weight per kernel from 4.7 to 2.7 mg, but even this difference is highly significant as the odds are 3.3×10^{17} to 1 against the probability that a deviation as large as, or larger than, the one indicated could have arisen from random sampling. In the comparison shown in table 2, of the kernels of the third floret, the kernels of the basal spikelet which were significantly below the average in weight and were not infected, are included. Omitting the kernels of the basal spikelet, the difference in weight is still 3.7 ± 0.66 mg. The difference in weight in the unpaired basal kernels is 2.5 ± 0.8 mg and is significant. Considering the 16 plants individually, the black-point kernels of 15 of the plants were the heavier, and the difference was significant in 9 of the 15 cases. In the exceptional case the healthy kernels had the greater weight, but the difference was not significant.

STUDY OF KERNEL WEIGHT ALONG THE SPIKE

In studying the paired kernels attention was given to the disposition of the black-point and healthy kernels along the spike. The results for the 16 plants are summarized in table 3.

TABLE 3.—*Distribution along the spike of black-point and healthy kernels, in pairs, the differences in kernel weight and percentage distribution of kernel weight*

Spikelet no.	Black-point				Healthy				Differences in weight between diseased and healthy kernels		Healthy kernels
	Kernels		Kernel weight per spikelet	Distribution of kernel weight	Kernels		Kernel weight per spikelet	Distribution of kernel weight			
	Number	*Per cent*	*Milligrams*	*Per cent*	*Number*	*Per cent*	*Milligrams*	*Per cent*	*Milligrams*	*Per cent*	*Percent*
1	27	7.9	35.7±0.55	7.2	145	14.0	32.9±0.30	12.7	2.9± .61	−5.5	84.4
2	52	15.1	40.1± .33	15.5	156	15.1	37.3± .26	15.5	2.8± .42	0	75.0
3	61	17.7	40.6± .33	18.5	157	15.1	39.2± .26	16.4	1.4± .42	2.1	72.1
4	75	21.8	41.0± .26	23.0	151	14.6	38.8± .27	15.6	2.2± .38	7.4	66.9
5	68	19.8	39.3± .33	19.9	148	14.3	38.0± .33	15.0	1.4± .47	4.9	68.5
6	39	11.3	35.4± .61	10.3	155	15.0	34.9± .34	14.4	.5± .70	−4.1	79.9
7	16	4.7	35.2±1.26	4.2	88	8.5	32.3± .44	7.6	2.9±1.34	−3.4	84.6
8	6	1.7	31.7±1.48	1.4	32	3.1	29.1± .77	2.5	2.6±1.67	−1.1	84.2
9	0	.0	.0	.0	4	.4	34.2	.4	....	−.4	100.0
Total or average	344	100.0	39.0± .17	100.0	1,036	100.1	36.2± .13	100.1	2.8± .21	−.1	....

From spikelet 1 (basal) to 6 the percentage distribution of the healthy kernels approaches uniformity, varying but 1.1 percent, while the distribution of black-point kernels over the same spikelets shows a range of 13.9 percent. The higher percentages for black-point kernels are found in the center of the spike, and it is there that the heavier healthy as well as black-point kernels are found. The aver-

age weights per kernel for each spikelet are multiplied by the corresponding percentage distributions, and these results, for the two groups, reduced to percentages, are found in columns 5 and 9. When the paired kernels in the two basal florets are studied, it is found that the heavier kernels are those which carry the greater amount of black point. The net results of this difference in weight are indicated in column 10.

The middle kernels of the spike are fertilized first, but the total time for blooming was quite certainly not over 3 days, as the weather was hot and dry. This earlier fertilization may have been responsible in part for a better nutrition of the central kernels and their consequent greater weight. The fungus may have found easier access to the ovules receiving the better nutrition. The time element may have been a factor influencing the place of infection on the spike, but it is doubtful whether the entire difference in weight can be attributed to this difference in time of infection.

It is seen in table 3 that differences in weight between the blackpoint and healthy kernels are found regularly in each spikelet. When the probable errors were calculated the differences were found to be statistically significant in the five lower spikelets. The few black-point kernels in 2 of the 3 upper spikelets resulted in probable errors too large for the differences to be significant although the absolute differences are relatively large. The sixth spikelet showed a small difference in weight in favor of the black-point kernels. The differences in the standard deviations are very generally contrary to those of the means, in that the distributions of the healthy kernels show greater variability than do those of the black-point kernels. The coefficients of variability of the two series, black point and healthy, for the eight spikelets are shown in table 4.

TABLE 4.—*Coefficients of variability of weights of the basal pair of kernels in the two groups, black point and healthy*

Spikelet no.	Coefficient of variability			Spikelet no.	Coefficient of variability		
	Black-point kernels	Healthy kernels	Difference		Black-point kernels	Healthy kernels	Difference
1	11. 70±1. 11	16. 44±. 67	4. 74±1. 30	5	10. 27±. 60	15. 93±. 64	5. 66±. 88
2	8. 66±. 58	12. 69±. 49	4. 03±. 76	6	15. 80±1. 25	17. 92±. 71	2. 12±1. 44
3	9. 32±. 57	12. 24±. 46	2. 92±. 73	7	20. 63±2. 56	18. 67±. 99	—1. 96±2. 75
4	8. 08±. 45	12. 46±. 49	4. 38±. 67	8	15. 42±3. 36	21. 90±. 97	6. 48±3. 51

In spikelets 1 to 5 the distribution of the healthy kernels has a variability significantly greater than that of the black-point kernels. If the two series of distributions are merged so that the distribution of the total healthy can be compared with the distribution of the total black point it becomes evident where the greater variability of the healthy-kernel series originates. The two distributions expressed in percentages are shown in table 5.

The sums of the positive and negative differences are necessarily equal. The healthy kernels show an excess of variates in the lower weight classes. This is suggested by the two means (table 2). The excess of distribution of the healthy kernels extends over 11 of the lower weight and 1 of the higher weight classes, while the correspond-

ing deficiency is confined to 6 classes in the higher ranges. Skewness is negative in both cases but more pronounced in the case of the healthy kernels. The greater variability shown in table 5 in the healthy kernels characterizes most of the spikelets and especially those which have the greater numbers of kernels.

TABLE 5.—*Percentages of healthy and black-point kernels in the various weight classes*

Item	Percentage of kernels in weight class indicated									
	16 to 17 mg	18 to 19 mg	20 to 21 mg	22 to 23 mg	24 to 25 mg	26 to 27 mg	28 to 29 mg	30 to 31 mg	32 to 33 mg	34 to 35 mg
Healthy kernels_____	0.4	0.6	1.2	1.5	2.4	4.0	4.1	6.7	8.0	11.1
Black-point kernels_	.3	.3	.0	.0	.6	.6	1.7	4.6	4.1	7.6
Difference_____	.1	.3	1.2	1.5	1.8	3.4	2.4	2.1	3.9	3.5

Item	Percentage of kernels in weight class indicated								
	36 to 37 mg	38 to 39 mg	40 to 41 mg	42 to 43 mg	44 to 45 mg	46 to 47 mg	48 to 49 mg	50 to 51 mg	Total
Healthy kernels_____	12.3	13.7	15.5	9.0	6.2	2.4	0.8	0.2	100.1
Black-point kernels_____	11.6	18.0	20.1	15.1	10.2	4.1	.6	.6	100.1
Difference_____	.7	−4.3	−4.6	−6.1	−4.0	−1.7	.2	−.4	.0

A study was made of the kernel weight of the first and second kernels per spikelet of spikes with nearly or quite healthy kernels. It was not evident that the lower kernel differed in weight from the kernel standing second in position. It follows, then, that the differentiation in weight of kernel with respect to incidence of black point upon the two lower kernels per spikelet is not due to the position of the kernel in the spikelet, associated with normal weight differences.

In any plant, on an average, the black-point kernels are heavier than those evidently free from disease. In part this weight difference may be ascribed to kernel position. Certain kernels of the spike are so located that their weight is decidedly less than that of kernels differently located. There is a differential in incidence of disease with regard to these two kernel groups. Thus weight differences of healthy and diseased kernels may be ascribed to weight differential due to locality in the spike, combined with a differential of disease incidence. But beyond this, between healthy and diseased kernels, weight differences are found which are not due to location in the spike, as is shown in table 2. Finally, a comparison, or series of comparisons, is to be found in the paired kernels of the basal florets of each spikelet. In each of the eight spikelets, the black-point kernels are the heavier (table 3). In these instances, also, the heavier weight of the black-point kernels is not due to any deviation from the normal in kernel size in conjunction with differences in incidence of disease.

The question arises whether the normally larger kernels of the spike were attacked by *Helminthosporium* (and *Alternaria*) or whether, when the young kernels of a group of potentially the same mature size were attacked, such kernels were somehow stimulated to develop a larger

amount of endosperm [7] than if no infection had taken place. It appears that both factors are responsible for the greater weight of the black-point kernels in the spike and in the plant.

CORRELATION RESULTS

It was possible to calculate correlation coefficients between percentages of black point and yield in the 1933 crop. In one instance a considerable number of yields were calculated from 5-foot rows. These rows were planted with F_5 selections from the cross Ceres × Hope-Florence; a single mother plant seeded one row. The stands of grain varied more or less; before harvest, notes were taken on the stand, and the yields were corrected to a uniform stand. The errors involved in this correction were thought to be less than those which would arise from uncorrected yields. Countings were made on the threshed samples of the frequency of the occurrence of black point and the percentages estimated. The weight per 1,000 kernels was also determined. The means and the standard deviations of these three characters calculated from 267 variates are shown in table 6.

TABLE 6.—*Correlations* [a] *between yield of wheat, weight of black-point kernels, and percentage of black-point kernels in the 1933 crop*

Item	Yield per acre	Weight of 1,000 kernels	Black-point kernels
	Bushels	*Grams*	*Percent*
Mean	34.45±0.31	37.14±0.09	21.98±0.48
Standard deviation	7.57± .22	2.06± .06	11.63± .34

[a] The 3 correlation coefficients calculated were: Black point and yield, 0.22±0.04; yield and 1,000-kernel weight, 0.32±0.04; black point and 1,000-kernel weight, 0.17±0.04.

None of these coefficients is very high, but the first one, which perhaps is of greatest interest, is about five times the probable error. Evidently there is a positive relationship between the presence of the infection and the yield, as secured. When the weight per 1,000 kernels is held constant the partial correlation between yield and black point is 0.18 ± 0.04. This still shows some significance.

In the cross first mentioned in this paper it was possible to calculate correlations similar to those given, except that weight of grain per plant was used instead of yield. The coefficients are as follows:

Black point and grain yield per plant ------------------------------ —0.09± 0.04
Yield per plant and 1,000-kernel weight ----------------------------- .27± .04
Black point and 1,000-kernel weight -------------------------------- .32± .03

When the weight per 1,000 kernels is held constant the correlation between black point and grain yield per plant becomes essentially zero. The total correlation between these characters is negative but not significant. Correlation of fairly high significance is shown between black point and 1,000-kernel weight. This is in keeping with the results shown in the earlier part of this paper.

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[7] As the endosperm in a normal kernel of wheat comprises about 85 percent of the total weight it is fair to presume that the excess weight of the diseased kernels would be distributed mainly to the endosperm. The black-point kernels appeared to be normal in shape. Any contribution to the greater weight of the black-point kernels made by the substance of the invading organism must have been negligible.

DISCUSSION

More exact studies as to the causal relationships with regard to the various external factors are greatly desired.   It is known that the epidemic of black point in 1933 was associated with high temperatures and low seasonal moisture conditions.   The wheats studied, started heading about June 22, and blossoming was probably well under way by June 25.   A comparison of temperatures and rainfall for the last 5 days of June and the first 15 days of July are shown in table 7.

TABLE 7.—*Temperatures and precipitation for the last 5 days of June and the first 15 days of July 1933*[a]

Item	June 26–30	July 1–5	July 6–10	July 11–15	Total
Temperature:	° F.	° F.	° F.	° F.	
Average daily mean	75	75	73	74	
Normal	67	68	67	67	
Excess in 1933	8	7	6	7	
Precipitation:	Inches	Inches	Inches	Inches	Inches
Total in 1933	1.65	.0	.81	.17	2.63
Normal	.86	.77	.47	.59	2.69

[a] Data are from the official records of the United States Weather Bureau.   The normal values of precipitation were taken from the Monthly Weather Review, Vol. 58, Suppl. No. 34, p. 59, May 1930.

The daily temperatures were greatly in excess of the normal for the whole 20 days and particularly during the last 5 days of June.   The season was unusually dry, but from June 26 to 30, 1.65 inches was recorded, which was decidedly in excess of the expected amount for the 5-day period.   Thus for a short period, in the early life of the wheat seed, conditions of high temperature and high rainfall obtained. It is quite unknown, of course, whether the weather conditions varied in such a manner during the period of infection as to account for the differences in incidence of black point which were shown to exist between basal kernels and kernels of the third floret and also between the mid-spike kernels and those near the base and near the tip of the spike.   It is not conceivable that any differentiation in weight due to position could be brought about within a single spikelet or in the third-floret kernels within the spike, as was the case in several instances.

On the other hand there seems to be no report in the literature that a seed infected by a fungus is stimulated, and consequently increases in growth.   If stimulation resulted in this case, an increased amount of endosperm must have been laid down, but there seems to be little or no information as to any causal relationship which might have brought this about.

SUMMARY

A heavy infection of black point, caused largely by *Helminthosporium sativum* Pam., King, and Bak., was found on various common wheats at Fargo in 1933.   On any one plant, in the hybrids studied, the black-point kernels generally were definitely heavier than the kernels showing no evidences of infection.

This difference in weight can be ascribed in part to a difference in infection of kernels differing normally in size because of position in the spike.   Third-floret kernels and end-spike kernels carried less infection than the heavier mid-spike kernels.

Within any kernel group of the spike, such as the third-floret group, the black-point kernels were significantly heavier than the noninfected kernels. The obvious conclusion seems to be that a portion of the weight differences results from a stimulation of the development of the endosperm following the entrance of the fungus into the developing ovule.

In one experiment, a coefficient of correlation of 0.22 ± 0.04 was found between the percentage of black point and the yield in 5-foot rows and a coefficient of 0.32 ± 0.04 was found between black point and weight of 1,000 kernels. In a study of individual plants resulting from a different cross, a correlation of -0.09 ± 0.04 was secured between the percentage of black point and the weight of the grain per plant and a correlation of 0.32 ± 0.03 was obtained between percentage of black point and the weight per 1,000 kernels.

A STATISTICAL STUDY OF THE RELATIONSHIPS BETWEEN THE CONSTITUENTS OF MILK [1]

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INTRODUCTION

The quantitative relationships of the fat, protein, total solids, and solids not fat in cow's milk have been extensively investigated by Overman, Sanmann, and Wright (*13*),[2] Gaines (*6*), Cranfield, Griffiths, and Ling (*4, 5*), and others. The purpose of this study is to contribute further to the subject in general, but especially to extend the consideration into the field of the inorganic constituents, which have received comparatively little attention.

Many studies of the relations existing between the different constituents of milk have been undertaken from the commercial point of view, especially with reference to the detection of adulteration, the establishment of legal standards, and the enrichment of milk, particularly in fat; and other studies have been made on the origin of the various constituents of milk, the methods of their secretion, the factors affecting their elaboration, and the interrelationship of these constituents. In the present study, which is statistical in method, the immediate objective has been to discover relationships of constituents which might subsequently be subjected to intensive investigation from the physiological point of view.

The data on which this paper is based were obtained from analyses of the milk of 12 Holstein-Friesian cows during an entire period of lactation. The animals were maintained under uniform conditions of care and feeding, without pasture, and were kept indoors, except for short daily periods of controlled exercise.

In relation to feeding treatment the cows were divided into two groups, one of which received timothy hay, corn silage, and concentrate mixture; the other, alfalfa hay, corn silage, and concentrate mixture. The protein content of the rations of the two groups was approximately equalized by varying the composition of the concentrates. Within both of the groups 2 cows were given a supplement of pulverized limestone, 2 a supplement of commercial bone meal, while 2 received no mineral supplement.

No evidence was found that the composition of the rations affected the composition of the milk in any characteristic manner. The analyses of the milk, therefore, were combined into a single group for the present study.

The usual seasonal variation in the composition of milk, dependent on changes in the ration, was not brought out by the method employed in this investigation, but the changes in the composition of milk which occur with the progress of lactation were observed, and these contributed to the statistical constants determined.

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The relationships reported are those of fat, energy, protein, total solids, solids not fat, lactose, ash, calcium, phosphorus, magnesium, chlorine, sodium, and potassium.

EXPERIMENTAL METHODS

The data treated represent 134 samples of milk which were, in greater part, composites of aliquots of each milking for 28-day periods. The time of calving, or of the end of the period of lactation, however, made certain of the milk-sampling periods shorter than the usual 28 days.

The method of preserving the milk, and the methods and results of the determinations of energy, fat, protein, total solids, and solids not fat, have been reported in a previous publication by Kahlenberg and Voris (*10*).

Ash, sodium, and potassium were determined by the official methods of the Association of Official Agricultural Chemists (*2*).

Calcium and magnesium were determined by the McCrudden method (*11, 12*), combined with the filtration and titration technic suggested by Halverson and Schulz (*8*).

Phosphorus was determined gravimetrically by the Neumann-Pemberton wet combustion method (*15*).

Chlorine was determined by the official method of the Association of Official Agricultural Chemists (*2*) except that 0.05 normal silver nitrate solution and 0.03 normal potassium thiocyanate were used instead of 0.1 normal silver nitrate and potassium thiocyanate as specified.

The percentage of lactose was obtained by subtracting the sum of the percentages of ash and protein from the percentage of solids not fat.

The statistical methods were, in general, standard procedures, in the form used by Harris and Benedict (*9*), with supplementary use of Pearson's tables (*14*).

PRESENTATION OF DATA AND DISCUSSION OF RESULTS

Table 1 summarizes the analytical data arranged in the order of increasing percentage of fat, by giving the mean percentage of each constituent corresponding to intervals of 0.2 percent in the mean fat content. The means, standard deviations, and coefficients of variation, with their respective probable errors, are given in table 2; the coefficients of correlation between the milk constituents are recorded in table 3; and the correlation coefficients are arranged with respect to magnitude and statistical significance, in the form of a frequency distribution, in figure 1.

VARIABILITY

It has been generally conceded in discussions of the composition of milk that fat is one of the most variable, if not the most variable, constituent. However, the individual inorganic constituents have not generally been taken into account—the ash usually being considered as a single ingredient—and the samples of milk have not usually been obtained from animals under conditions of experimental control.

The coefficients of variation in table 2 show that the sodium, the chlorine, and the magnesium content of milk varies much more than

does the fat, and that protein varies as much as does fat. It may be that the fat content of milk is more easily affected by disturbing conditions than is the protein content, and that under the uniform conditions maintained throughout this experiment fat was less variable than it is under ordinary feeding practices.
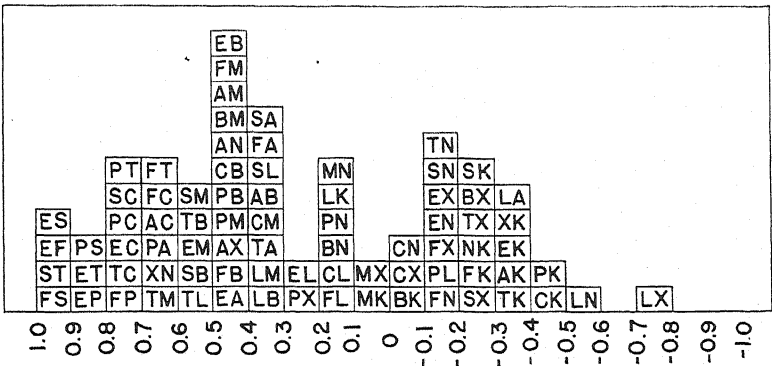
FIGURE 1.—Distribution of correlation coefficients between quantities of milk constituents: *F*, fat; *E*, energy; *P*, protein; *S*, total solids; *T*, solids not fat; *L*, lactose; *A*, ash; *C*, calcium; *B*, phosphorus; *M*, magnesium; *X*, chlorine; *N*, sodium; and *K*, potassium.

TABLE 1.—*Summary of analytical data, giving average percentages of each milk constituent when mean fat content is aranged in 0.2 percent classes*

Fat range (percent)	Samples	Fat	Nitrogen	Protein	Total solids	Solids not fat	Energy per gram	Lactose
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Calories*	*Percent*
2.60 to 2.79	6	2.70	0.424	2.71	10.85	8.15	586	4.71
2.80 to 2.99	13	2.93	.434	2.77	11.37	8.44	615	4.93
3.00 to 3.19	26	3.10	.447	2.85	11.79	8.69	643	5.12
3.20 to 3.39	34	3.29	.470	3.00	12.08	8.79	666	5.06
3.40 to 3.59	13	3.46	.498	3.25	12.46	9.00	690	5.01
3.60 to 3.79	18	3.72	.510	3.31	12.68	8.96	716	4.93
3.80 to 3.99	10	3.89	.513	3.27	12.98	9.09	732	5.05
4.00 to 4.19	7	4.09	.569	3.63	13.49	9.40	774	5.01
4.20 to 4.39	3	4.25	.571	3.65	13.93	9.68	793	5.24
4.40 to 4.60	4	4.50	.600	3.83	14.40	9.90	825	5.26

Fat range (percent)	Samples	Ash	Sodium	Potassium	Calcium	Magnesium	Phosphorus	Chlorine
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
2.60 to 2.79	6	0.742	0.081	0.143	0.094	0.013	0.085	0.132
2.80 to 2.99	13	.741	.063	.146	.103	.012	.091	.110
3.00 to 3.19	26	.737	.054	.149	.106	.012	.093	.100
3.20 to 3.39	34	.743	.055	.143	.109	.013	.094	.108
3.40 to 3.59	13	.770	.076	.140	.116	.013	.097	.117
3.60 to 3.79	18	.775	.058	.128	.121	.013	.097	.109
3.80 to 3.99	10	.768	.068	.144	.115	.015	.101	.098
4.00 to 4.19	7	.768	.059	.126	.123	.014	.095	.110
4.20 to 4.39	3	.790	.082	.121	.130	.016	.105	.109
4.40 to 4.60	4	.810	.066	.139	.128	.019	.111	.095

Among the individual inorganic constituents, sodium seems to be the most variable. This may be only apparent, the variability of the data being due in part to difficulties encountered in analysis; but chlorine, with which sodium shows the closest correlation, and which is not particularly difficult to determine, also shows a variability

strikingly greater than that of any of the other constituents except magnesium. Therefore, inasmuch as sodium and chlorine are largely in combination with each other, sodium chloride is presumably the most variable constituent of milk. Possible explanations of this variability are suggested in the discussion of the correlation coefficients.

TABLE 2.—*Means, standard deviations, and coefficients of variation of constituents of milk*

Constituent	Means	Standard deviations	Coefficients of variation
Fat	3.4095±0.0245	0.4207±0.0173	12.34±0.52
Protein	3.0770±.0224	.3838±.0158	12.47±.52
Total solids	12.2713±.0481	.8250±.0340	6.72±.28
Solids not fat	8.8619±.0277	.4752±.0196	5.36±.22
Energy	680.075±3.299	56.61±2.332	8.32±.35
Lactose	5.0274±.0181	.3108±.0128	6.18±.26
Ash	.7547±.0024	.0420±.0017	5.57±.23
Sodium	.0576±.0011	.0184±.0008	31.94±1.44
Potassium	.1392±.0010	.0172±.0007	12.36±.52
Calcium	.1117±.0007	.0117±.0005	10.47±.44
Magnesium	.0131±.0002	.0030±.0001	22.90±.99
Phosphorus	.0951±.0005	.0090±.0004	9.46±.39
Chlorine	.1077±.0013	.0216±.0009	20.06±.86

TABLE 3.—*Coefficients of correlation between quantities of milk constituents*

Constituent	Correlation r	Constituent	Correlation r
Fat and—		Total solids and—Continued.	
Energy	0.9502±0.0057	Phosphorus	0.5448±0.0410
Total solids	.9076±.0103	Ash	.3843±.0497
Protein	.7000±.0297	Lactose	.3667±.0504
Solids not fat	.6885±.0306	Sodium	-.1555±.0569
Calcium	.6646±.0325	Chlorine	-.2064±.0558
Magnesium	.4690±.0455	Potassium	-.2932±.0533
Phosphorus	.4166±.0482	Solids not fat and—	
Ash	.3823±.0498	Total solids	.9382±.0070
Lactose	.1264±.0573	Energy	.8565±.0155
Sodium	-.1027±.0576	Protein	.7808±.0222
Chlorine	-.1194±.0574	Calcium	.7670±.0240
Potassium	-.2242±.0553	Fat	.6885±.0306
Energy and—		Magnesium	.6083±.0367
Total solids	.9817±.0021	Phosphorus	.5813±.0386
Fat	.9502±.0057	Lactose	.5320±.0418
Solids not fat	.8565±.0155	Ash	.3362±.0517
Protein	.8101±.0200	Sodium	-.1777±.0564
Calcium	.7698±.0237	Chlorine	-.2506±.0546
Magnesium	.5612±.0399	Potassium	-.3061±.0528
Phosphorus	.4787±.0449	Lactose and—	
Ash	.4125±.0484	Solids not fat	.5320±.0418
Lactose	.2491±.0547	Total solids	.3667±.0504
Sodium	-.1201±.0574	Magnesium	.3281±.0520
Chlorine	-.1452±.0570	Phosphorus	.3005±.0530
Potassium	-.3252±.0521	Energy	.2491±.0547
Protein and—		Potassium	.1767±.0565
Total solids	.8760±.0136	Calcium	.1306±.0573
Energy	.8101±.0200	Fat	.1264±.0573
Solids not fat	.7868±.0222	Protein	-.1134±.0575
Calcium	.7728±.0235	Ash	-.3891±.0494
Fat	.7000±.0297	Sodium	-.5007±.0437
Ash	.6194±.0359	Chlorine	-.7030±.0295
Phosphorus	.4354±.0472	Ash and—	
Magnesium	.4262±.0477	Calcium	.06378±.0346
Chlorine	.2197±.0555	Protein	.6194±.0359
Sodium	.1445±.0571	Magnesium	.4579±.0460
Lactose	-.1134±.0575	Sodium	.4511±.0464
Potassium	-.4934±.0441	Chlorine	.4185±.0481
Total solids and—		Energy	.4125±.0484
Energy	.9817±.0021	Total solids	.3843±.0497
Solids not fat	.9382±.0070	Fat	.3823±.0498
Fat	.9076±.0103	Phosphorus	.3564±.0509
Protein	.8760±.0136	Solids not fat	.3362±.0517
Calcium	.7793±.0229	Potassium	-.3219±.0522
Magnesium	.5893±.0380	Lactose	-.3891±.0494

TABLE 3.—*Coefficients of correlation between quantities of milk constituents*—Con.

Constituent	Correlation *r*	Constituent	Correlation *r*
Calcium and—		Chlorine and—	
Total solids	0.7793±0.0229	Sodium	0.6109±0.0365
Protein	.7728± .0235	Ash	.4185± .0481
Energy	.7698± .0237	Protein	.2197± .0555
Solids not fat	.7670± .0240	Magnesium	.0444± .0582
Fat	.6646± .0325	Calcium	−.0620± .0580
Ash	.6378± .0346	Fat	−.1194± .0574
Phosphorus	.4508± .0464	Energy	−.1452± .0570
Magnesium	.3365± .0517	Total solids	−.2064± .0558
Lactose	.1306± .0573	Solids not fat	−.2506± .0546
Chlorine	−.0620± .0580	Phosphorus	−.2632± .0542
Sodium	−.0785± .0579	Potassium	−.3825± .0497
Potassium	−.4399± .0470	Lactose	−.7030± .0295
Phosphorus and—		Sodium and—	
Solids not fat	.5813± .0386	Chlorine	.6109± .0365
Total solids	.5448± .0410	Ash	.4511± .0464
Energy	.4787± .0449	Magnesium	.1926± .0561
Magnesium	.4519± .0464	Protein	.1445± .0571
Calcium	.4508± .0464	Phosphorus	.1383± .0572
Protein	.4354± .0472	Calcium	−.0785± .0579
Fat	.4166± .0482	Fat	−.1027± .0576
Ash	.3564± .0509	Energy	−.1201± .0574
Lactose	.3005± .0530	Total solids	−.1555± .0569
Sodium	.1383± .0572	Solids not fat	−.1777± .0564
Potassium	−.0095± .0583	Potassium	−.2367± .0550
Chlorine	−.2632± .0542	Lactose	−.5007± .0437
Magnesium and—		Potassium and—	
Solids not fat	.6083± .0367	Lactose	.1767± .0565
Total solids	.5893± .0380	Magnesium	.0382± .0582
Energy	.5612± .0399	Phosphorus	−.0095± .0583
Fat	.4690± .0455	Fat	−.2242± .0553
Ash	.4579± .0460	Sodium	−.2367± .0550
Phosphorus	.4519± .0464	Total solids	−.2932± .0533
Protein	.4262± .0477	Solids not fat	−.3061± .0528
Calcium	.3365± .0517	Ash	−.3219± .0522
Lactose	.3281± .0520	Energy	−.3252± .0521
Sodium	.1926± .0561	Chlorine	−.3825± .0497
Chlorine	.0444± .0582	Calcium	−.4399± .0470
Potassium	.0382± .0582	Protein	−.4934± .0441

The exceptional variability of magnesium which was observed has also been noted by Allen (*1*), but no adequate explanation of this fluctuation is apparent.

Solids not fat show the least variability, followed, in the order of increasing variability, by ash, lactose, total solids, energy, phosphorus, calcium, chlorine, magnesium, and sodium.

CORRELATIONS

In the interpretation of the correlation coefficients any correlations greater than 0.4600, that is, correlations of a magnitude greater than 10 times their probable error, have been considered of definite significance. Correlations of from 0.3130 to 0.4600, which are more than 6 and less than 10 times their probable error, have been designated as of doubtful significance, and correlations of less than 6 times their probable error have not been considered significant.

The following general statements of significant relationships were observed:

(1) As the percentage of fat increases, the energy, and the percentages of total solids, protein, solids not fat, calcium, and magnesium increase.

(2) As the energy value increases, the percentages of total solids, solids not fat, protein, calcium, magnesium, and phosphorus increase.

(3) As the percentage of protein increases, the percentages of total solids, solids not fat, calcium, and ash increase, and the percentage of potassium decreases.

(4) As the percentage of total solids increases, the percentages of solids not fat, calcium, magnesium, and phosphorus increase.

(5) As the percentage of solids not fat increases, the percentages of calcium, magnesium, phosphorus, and lactose increase.

(6) As the percentage of lactose increases, the percentages of sodium and chlorine decrease.

(7) As the percentage of ash increases, the percentage of calcium increases.

(8) As the percentage of chlorine increases, the percentage of sodium increases.

Among the correlations of doubtful significance it is of interest to note that as magnesium increases the percentages of ash, phosphorus, protein, calcium, and lactose increase. It has been noted above that magnesium is significantly correlated with solids not fat, total solids, energy, and fat. Thus, in 9 of the 12 comparisons, magnesium shows a significant, or probably significant, positive correlation. Similarly, calcium shows high positive correlations with total solids, protein, energy, solids not fat, fat, and ash, with possibly significant positive correlations with phosphorus and magnesium.

Among the negative correlations of special interest are those of potassium with protein and calcium; and of lactose, which is a relatively constant constituent, with sodium and chlorine, which are extremely variable. The findings with reference to lactose, sodium, and chlorine are in harmony with the conclusion of Porcher (16) that milk with high lactose content is usually low in sodium chloride, and that mainly by variation in the sodium chloride in the milk it is maintained isotonic with the blood.

Gowen and Tobey (7) state that the osmotic pressure of milk is very largely determined by the lactose content, whereas the osmotic pressure of blood is relatively little determined by its sugar content but mostly by its salt content, referring to six major osmotically active elements, potassium, sodium, calcium, magnesium, phosphorus, and chlorine. In the maintenance of the isotonicity of blood and milk, the milk of a lower lactose content would have to increase its content of salts in a rather pronounced degree to make up for this deficiency of lactose. Gowen and Tobey propose to compute the osmotic pressure from the millimolar concentration of lactose and ash found in the milk. From the present data lactose does reveal a negative correlation with ash, but among the individual elements only sodium and chlorine were found negatively correlated with lactose.

Blackwood and Stirling (3) consider that by far the largest part of any osmotic change taking place during milk secretion is to be accounted for by the synthesis of protein and carbohydrate from glucose and amino acids. They consider that during milk secretion a transudate is separated from the blood, having the same osmotic pressure as blood, and that during synthesis water is returned to the blood to maintain the tonicity of the cell fluid.

In the present study protein shows no significant correlations, positive or negative, with lactose and sodium chloride; and these correlations, as contrasted with the significantly negative correlation

of lactose and sodium chloride, suggest a dissimilar relation of protein and lactose in the maintenance of the isotonicity of the blood and milk.

The narrow range of variation in the hydrogen-ion concentration of freshly drawn milk, between 6.50 and 6.65, is probably maintained chiefly through the phosphates and casein present. It was shown by Rice and Markley (*17*) that the hydrogen-ion concentration of milk of high acidity, as determined by titration, is affected by the addition of acid or alkali to a much lesser extent than is the hydrogen-ion concentration of milk with low acidity. Their explanation is that in high-acid milk the phosphate and casein are present in greater quantities than in low-acid milk, and that the buffer action is greater in the former case than in the latter. The negative correlation of potassium and protein is in harmony with this conclusion, and since calcium is largely associated with protein the negative correlation of potassium and calcium seems to be a part of the same picture. However, there is no correlation, either positive or negative, between potassium and phosphorus. In fact, potassium shows no significant positive correlation with any of the constituents determined, though it may be correlated with undetermined constituents, for instance, with citric acid.

Wright (*18*) has shown experimentally that $CaHPO_4$ in solutions of calcium caseinate may be held in stable colloidal solutions up to the concentrations of calcium and phosphorus found in milk. He suggests that the calcium and phosphorus which diffuse from the blood into the mammary gland may thus be held in colloidal combination by the casein in the milk cells. Alkali renders this combination unstable, with which fact the negative correlations of potassium with protein and calcium, as found in this study, seem to be in harmony. This calls attention to the slight degree of correlation found between potassium and phosphorus, and suggests that phosphorus is associated with other constituents than calcium and casein—which is borne out by the fact that the ratio of phosphorus to calcium in calcium caseinate is 0.8, and in $CaHPO_4$ it is 0.775, whereas the ratio between the average percentages of phosphorus to calcium found in milk in the present study is 0.851.

In consideration of the questionable significance of correlations as evidence however, no effort will be made to interpret these observations closely. Correlations can only suggest, and the facts as to origin and relationships of the constituents of milk must be established by evidence of more direct and less equivocal character.

SUMMARY

Statistical data based on the analyses of 134 samples of Holstein-Friesian milk representing one entire lactation period for each of 12 cows are reported. The analyses include fat, energy, protein, total solids, solids not fat, lactose, ash, calcium, phosphorus, magnesium, chlorine, sodium, and potassium. The means, standard deviations, and coefficients of variation of each constituent with their corresponding probable errors, are reported, and the coefficients of correlation of each constituent with every other constituent are given.

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INFLUENCE OF CALCIUM PHOSPHORUS INTAKE ON BOVINE BLOOD[1]

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INTRODUCTION

Phosphorus is present in every cell and fluid of the body. It is vitally concerned with carbohydrate, fat, and some phases of protein metabolism. It is essential in muscular contractions and in the functioning of the central nervous system. It enters into the buffering powers of the blood and other tissues. Phosphorus, together with calcium, plays a fundamental role in bone formation. It is, then, not surprising to find profound physiological and anatomical disturbances occurring in animals on a phosphorus-deficient diet. In cattle such a deficiency may manifest itself by hypophosphoraemia, osteophagia, and osteoporosis, and by osteomalacia in the adult (*15*).[2] In the early stages the consumption of food is usually adversely affected (*26*). On phosphorus-deficient rations the milk yield is usually low (*2*) and there is a marked inhibition of oestrum (*4*). Usually before most of the symptoms appear, hypophosphoraemia manifests itself (*3*, *24*).

Aphosphorosis of cattle is known to occur in many regions (*5*, *25*). It is probably even more widely spread than is known at the present time, where the following conditions occur: The forage is produced on phosphorus-deficient soil and consequently is low in phosphorus; the cattle are kept on too restricted rations, the main constituents of which are deficient in phosphorus; the intake of calcium is excessive in relation to the intake of phosphorus; and the phosphorus require-ments are extremely high and hence not met by the ordinary rations, as in the case of the lactating cow.

It has been suggested that the estimation of inorganic blood phos-phorus gives an early diagnosis of aphosphorosis and helps to differen-tiate it from picas having a different origin (*14*, *26*). With sufficient information on the optimum inorganic phosphorus of the blood under varying conditions, it appears probable that blood analyses may be used to discover those animals which are on phosphorus-deficient diets, and hence the proper phosphorus supplement may be prescribed. In this manner, at least in a degree, it may be possible to prevent the modern dairy cow from passing through a mild form of osteoporosis at each lactation. This is an extreme instance and at times may not be due to a deficient ration but rather to an overdeveloped udder outrunning the absorptive capacity of the gut for phosphorus (*6*). However, Huffman and his coworkers (*12*) found that even high-yielding cows (2,000 gallons) can be maintained in mineral equilibrium (positive calcium balance) upon ordinary mineral-rich rations. Nevertheless, the margin is often exiguous, and little may be required to change a positive balance to a negative one.

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EXPERIMENTAL ANIMALS AND PROCEDURE

Data reported in this paper represent blood analyses obtained from 40 grade yearling beef steers, averaging 585 pounds in weight and of similar breeding and previous treatment. They were carefully sorted into five groups as nearly alike as possible as to weight, quality, and condition. They were fed for a period of 150 days on the following rations:

Lot 1, pressed beet pulp, beet molasses, alfalfa hay, and salt.
Lot 2, pressed beet pulp, beet molasses, alfalfa hay, and cottonseed cake.
Lot 3, pressed beet pulp, beet molasses, alfalfa hay, and steamed bone meal.
Lot 4, pressed beet pulp, beet molasses, alfalfa hay, and mill-run bran.
Lot 5, pressed beet pulp, beet molasses, alfalfa hay, and ground barley.

The average daily ration consumed by each lot, the daily calcium and phosphorus intakes, and the calcium-phosphorus ratio in the rations are shown in table 1.

Lot 1, receiving beet pulp, molasses, alfalfa, and salt, obtained less than 50 percent of the phosphorus received by those on the ration supplemented with cottonseed cake. The phosphorus intake of lot 5, where barley was given, was also low; that of the remaining lots was high. There were also considerably greater quantities of calcium in the rations of the animals receiving phosphorus supplements. This is especially noticeable in the lot receiving cottonseed cake. The calcium oxide intake in the ration of lot 1 equaled 0.74 percent; of lot 2, 0.69 percent; of lot 3, 0.48 percent; of lot 4, 0.52 percent; and of lot 5, 0.73 percent. All these animals received rations containing more than 0.45 percent of calcium oxide placed as the minimum by the Wisconsin workers (10) for dry dairy cows. Phosphorus was low in the rations of lots 1 and 5 when compared with the quantities recommended by Kellner (13) and Armsby (1).

The animals receiving no phosphorus supplements were getting considerably less than the 30 g of phosphorus pentoxide per day, which Theiler, Green, and Du Toit (27) laid down as the minimum for heifers. Where barley was the supplement, the animals were receiving 30 g; all others were receiving considerably more than the minimum requirements.

Theiler, Green, and Du Toit (26) conclude that inasmuch as minimum requirements of growth are higher in the case of phosphorus than in the case of calcium, a ratio of calcium oxide to phosphorus pentoxide as high as 3 to 1 is not necessarily disadvantageous. Lots 2 and 3 were receiving rations in which the ratio of calcium oxide to phosphorus pentoxide was 3 to 1. Lot 1 received calcium oxide to phosphorus pentoxide in a ratio of 4.6 to 1 and lot 5 in the ratio of 4.1 to 1. It is evident, therefore, that in lots 1 and 5 phosphorus could have been profitably added. One is prone to ask: Could greater quantities of phosphorus have been added advantageously to the rations of lots 2, 3, and 4? Theiler, Green, and Du Toit (26) state that when calcium is low it is probable that a relatively high proportion of phosphorus may facilitate the absorption of calcium and thus reduce the risk of calcium starvation. When phosphorus is low, a relatively high calcium intake may reduce the absorption of phosphorus and in this way increase the danger of aphosphorosis.

TABLE 1.—*Average daily ration consumed, average daily intake of calcium and phosphorus, and the calcium-phosphorus ratio in the rations*

Lot no.	1	2	3	4	5
Average ration consumed_____pounds	Beet pulp_____ 21.4 Molasses_____ 2.5 Alfalfa_____ 7.1 Salt_____ .09	Cottonseed cake 1.8 Beet pulp_____ 34.6 Molasses_____ 2.5 Alfalfa_____ 6.4 Salt_____ .06	Bone meal_____ 0.1 Beet pulp_____ 48.6 Molasses_____ 2.5 Alfalfa_____ 6.3 Salt_____ .04	Mill-run bran____ 2.7 Beet pulp_____ 42.0 Molasses_____ 2.5 Alfalfa_____ 6.3 Salt_____ .04	Ground barley__ 3.6 Beet pulp_____ 24.7 Molasses_____ 2.5 Alfalfa_____ 6.7 Salt_____ .04
Phosphorus as P_2O_5 in ration_____grams	23	48	41	48	30
Calcium as CaO in ration_____do	105	143	127	127	125
$\dfrac{CaO}{P_2O_5}$_____ratio	4.6	3.0	3.1	2.6	4.1

Samples of blood were collected in the morning before feeding to obviate the action of recently ingested carbohydrates on blood inorganic phosphorus. It was found in preliminary tests in 1932, when this precaution was not taken, that the blood inorganic phosphorus often fell to an extremely low level, probably due to the large quantities of soluble carbohydrates consumed.

The blood was collected from the jugular vein in two 4-ounce bottles. The sample for phosphorus determination was quickly mixed with sodium oxalate, immediately taken to the laboratory, and the plasma centrifuged out. The phosphorus was determined in the plasma by the Youngberg method (*11*); the calcium in the serum of the second sample by the Clark-Collip modification of the Krammer-Tisdal method (*11*). Results are reported as milligrams of phosphorus in 100 cc of blood plasma and milligrams calcium in 100 cc of blood serum (table 2). Samples were taken at the beginning of the experiment and during each succeeding month. This work represents analyses of individual samples and not the composite of three samples taken on successive days as reported by some workers. In this work, however, samples were taken from each of eight animals receiving the same treatment. This should eliminate, to a degree, individual and daily fluctuations in the calcium and phosphorus content of the blood.

EXPERIMENTAL DATA

The average calcium content of the blood of the various lots on January 21, 1933, varied from 12.25 mg per 100 cc of blood serum to 13.16 mg. With few exceptions, there is only a small variation within the different groups (table 2).

TABLE 2.—*Milligrams of calcium per 100 cc of blood serum and of inorganic phosphorus per 100 cc of blood plasma in blood of steers fed different supplements to the ration, Jan. 21 and Feb. 28, 1933*

DATA OF JAN. 21, 1933

Steer no.	Lot 1, no supplement		Lot 2, cottonseed cake			Lot 3, steamed bone meal			Lot 4, mill-run bran			Lot 5, ground barley		
	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P
3	13.50	2.87	1	12.00	2.30	2	12.00	1.89	7	13.50	2.56	5	13.25	2.52
12	12.00	2.71	6	13.00	2.18	4	11.25	2.68	13	12.00	3.16	8	11.75	3.76
22	14.00	3.07	10	11.75	2.47	16	13.50	3.65	21	12.50	2.72	23	12.00	2.78
29	13.75	2.26	15	13.75	2.15	18	13.50	3.40	24	14.00	1.74	28	13.25	2.91
40	13.75	2.54	25	14.00	1.67	32	11.00	3.51	26	11.25	2.93	31	12.50	3.46
41	12.00	2.59	30	13.00	3.10	33	11.25	2.75	34	11.75	2.38	36	10.50	2.96
46	11.50	2.79	42	13.75	2.21	14	12.00	2.47	35	14.00	1.69	44	13.00	3.21
49	13.75	2.24	43	13.75	3.23	48	16.00	2.72	45	13.75	3.73	50	11.75	2.49
Average	13.04	2.63		13.13	2.41		12.56	2.88		12.84	2.61		12.25	3.01

DATA OF FEB. 28, 1933

Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P
3	13.3	3.13	1	13.5	4.49	14	13.3	4.74	21	14.0	6.67	50	15.8	3.38
12	13.0	3.25	25	14.3	4.07	48	13.5	6.25	45	14.0	4.55	44	14.0	4.35
22	13.0	4.13	6	13.8	3.54	2	12.8	6.29	34	14.8	4.52	5	15.3	3.02
29	15.0	3.13	43	13.5	4.55	18	12.8	5.71	13	14.0	7.04	23	14.5	3.37
40	13.5	3.73	15	14.0	4.07	18	14.5	4.17	24	14.5	4.39	8	14.8	4.42
41	12.8	3.70	42	14.3	5.38	33	14.3	5.38	26	11.3	5.95	36	15.3	3.31
46	14.0	3.91	10	13.0	4.39	32	13.0	6.58	35	14.5	3.57	28	14.0	3.16
49	13.0	3.85	30	14.0	4.52	4	13.5	5.65	7	14.0	6.62	31	15.3	4.35
Average	13.4	3.60		13.8	4.38		13.5	5.60		13.9	5.41		14.9	3.67

These animals had grazed on the range plants which were grown on the highly calcareous soils of Idaho and consequently were high in calcium. This may account for the high calcium and comparatively low phosphorus content of the animal's blood.

The inorganic phosphorus of the blood varied from 2.41 to 3.01 mg per 100 cc of blood plasma. These results are low when compared with those obtained by Eckles and his coworkers (5) on animals receiving sufficient phosphorus and point strongly to the conclusions that all these animals, although on a ration high in alfalfa hay grown on phosphorus-rich soils, would have benefited if given a phosphorus supplement.

The phosphorus-calcium ratio in the blood varied from 1 to 4 in barley-fed steers and from 1 to 5.5 in animals receiving cottonseed cake.

On February 28, 1933, the blood was again analyzed (table 2). A slight increase in the blood calcium had occurred. Even where the animals received the calcium supplements there was an increase of only 1 mg per 100 cc of blood serum, which is in keeping with the findings of Theiler, Green, and Du Toit (26). The phosphorus, however, had markedly increased. The animals receiving bone meal and mill-run bran yielded blood phosphorus well above 5 mg per 100 cc of blood plasma. In those animals receiving no phosphorus supplement and those receiving ground barley, the blood phosphorus was lower, being 3.60 and 3.67 mg per 100 cc of blood plasma, respectively. The phosphorus-calcium ratio, while still varying widely in the blood of different lots, was much narrower.

Blood samples were also taken on April 1, April 27, and June 1, 1933. These showed the same regularity in each group, as did the analyses made on January 21 and February 28. Hence, only the averages of these determinations are given (table 3). Each reported result represents the averages of the analyses made on eight different animals receiving the same ration which should rule out individual differences that occur when averages are made from individual animals.

TABLE 3.—*Average milligrams of calcium in 100 cc of blood serum and of inorganic phosphorus per 100 cc of blood plasma in the blood of steers fed different supplements to the ration on given dates*

[Averages represent 8 determinations made on different animals receiving the same rations]

CALCIUM

Date of sampling	Lot 1, no supplement	Lot 2, cottonseed cake	Lot 3, steamed bone meal	Lot 4, mill-run bran	Lot 5, ground barley
1933					
Jan. 21	13. 16	13. 13	12. 58	12. 84	12. 25
Feb. 28	13. 40	13. 18	13. 50	13. 90	14. 90
Apr. 1	15. 22	12. 03	12. 31	12. 63	12. 56
Apr. 27	13. 41	12. 19	12. 19	11. 88	12. 59
June 1	14. 10	13. 00	13. 00	12. 20	12. 80

INORGANIC PHOSPHORUS

Date of sampling	Lot 1, no supplement	Lot 2, cottonseed cake	Lot 3, steamed bone meal	Lot 4, mill-run bran	Lot 5, ground barley
1933					
Jan. 21	2. 63	2. 41	2. 88	2. 61	3. 01
Feb. 28	3. 60	4. 38	5. 60	5. 41	3. 67
Apr. 1	2. 67	4. 12	5. 22	4. 52	3. 58
Apr. 27	2. 60	4. 28	4. 71	4. 52	3. 35
June 1	3. 11	4. 45	4. 35	4. 98	3. 73

When placed on the experimental ration, these cattle were taken from feeds high in calcium. Moreover, all experimental rations carried calcium well above the minimum requirements for such animals; consequently, their blood calcium showed no appreciable variation that could be ascribed to these rations. The greatest variation is shown in the lot receiving no phosphorus supplement. Due to the drop in phosphorus, nature may have attempted to maintain an approximate uniform calcium times phosphorus concentration. Palmer and Eckles (*20*) produced hypercalcemia and hypophosphoraemia in cows by feeding diets low in phosphorus. Had the animals used in the experiments here reported been younger, it is highly probable that greater variations would have occurred in the serum calcium, for it is not easy to raise the concentration of serum calcium far above normal by feeding calcium salts or high calcium-containing feed; it is even more difficult to lower the serum calcium of normal full-grown animals by altering the intake of calcium (*23, v. 1, p. 814*).

The inorganic phosphorus of the blood plasma on January 21, when the cattle were placed on the various rations, was low. There was an increase on February 28, greatest in the case of steers receiving bone meal and least where no phosphorus supplement was given. The concentrations remained quite uniform throughout the remainder of the experiment. Only occasionally did the inorganic phosphorus content reach 5-mg concentration, making it highly probable that all of these animals would have benefitted by an increase in the phosphorus supplement due to the high calcium intake.

DISCUSSION

It has been stated (*23*) that calcium and phosphorus in the circulating plasma, other factors remaining constant, bear a reciprocal relationship to one another. Harnes (*8, 9*) tested this biometrically on the blood of rabbits and found a small negative correlation between the calcium and inorganic phosphorus. Palmer and his coworkers (*21*) found no correlation between calcium and inorganic phosphorus and consider Harnes' coefficient so small in relation to the probable error as to be mathematically insignificant. Calculations have been made of the correlation coefficients between calcium and phosphorus in the 199 analyses, as reported herein. The formulas given by Pearl (*22*)

were used: $r_{12} = \dfrac{(x_1 x_2)}{N \sigma_1 \sigma_2}$ and $P.E._r = 0.67449 \dfrac{1 - r^2}{\sqrt{N}}$.

The values for r_{12} on the following dates are:

Jan. 21, -0.1660 ± 0.1037 ($n=40$)
Feb. 28, -0.3333 ± 0.0947 ($n=40$)
Apr. 1, -0.5116 ± 0.0784 ($n=40$)
Apr. 27, -0.5888 ± 0.0696 ($n=40$)
June 1, -0.6096 ± 0.0678 ($n=39$)
On all samples, -0.2264 ± 0.0452 ($n=199$)

Hence, there is a small negative correlation between the inorganic phosphorus and calcium in the blood of steers fed on the rations considered in this work. It becomes greater as the experiment progresses; however, more work is necessary before it can be said to have a biological significance.

There is a close relationship between the phosphorus intake of these animals and the inorganic phosphorus of the blood plasma, which bears out the findings of others (*11, 19*). Analyses of blood for its inorganic phosphorus content can be used as a measure to determine the phosphorus needs of animals. This, however, requires further work to standardize the factor for optimum concentration under varying conditions. The age of the animal is known to influence the inorganic phosphorus of the blood (*7*), and the conclusion is quite well-founded that gestation is without effect (*16, 18*). However, the effect of sex and pregnancy upon the normal inorganic phosphorus of the blood is not so well known.

At the end of the 150-day period, the ration of the cattle receiving pressed beet pulp, beet molasses, alfalfa hay, and salt (lot 1) was supplemented with 0.1 pound of steamed bone meal daily. After being on this ration for 30 days, blood samples were taken and analyzed for calcium and inorganic phosphorus. These results are given in table 4.

TABLE 4.—*Milligrams of calcium in 100 cc of blood serum and of inorganic phosphorus in 100 cc of blood plasma in the blood of steers, July 1, 1933.*

Steer no.	Calcium	Phosphorus	Steer no.	Calcium	Phosphorus	Steer no.	Calcium	Phosphorus
40	13.3	3.4	3	14.5	2.4	50	13.0	2.4
6	12.5	3.9	49	13.3	2.0	22	12.5	3.5
34	12.5	3.3	46	12.0	3.9	5	13.0	3.3
12	13.0	3.4						

The calcium of the blood remained quite constant. The inorganic phosphorus of the blood increased; at the close of the experiment, however, it was still far below that of the animals which had received steamed bone meal from the beginning of the experiment. Undoubtedly, due to the deficient ration, other tissues of the body, in addition to the blood, had been depleted of their phosphorus; consequently, time apparently is required to bring the tissues back to normal.

SUMMARY

Forty head of grade beef steers, averaging 585 pounds in weight and of similar breeding and previous treatment, were sorted into 5 groups as nearly alike as possible as to weight, quality, and condition and were fed for 150 days on rations of pressed beet pulp, beet molasses, alfalfa hay, and salt, to which was added for four of the groups a supplement of cottonseed cake, steamed bone meal, mill-run bran, and ground barley, respectively.

Blood samples were taken monthly and analyzed for calcium and phosphorus. Before going on these rations the averages per 100 cc of blood serum and plasma were: Calcium from 12.25 to 13.13 mg and inorganic phosphorus from 2.41 to 3.01 mg. Phosphorus supplements fed in the form of cottonseed cake, steamed bone meal, and mill-run bran increased the blood inorganic phosphorus to near that reported as optimum by some workers or 5 mg per 100 cc of blood serum. It was lowest in the lot receiving ground barley and highest where steamed bone meal was fed. The various phosphorus supplements produced little, if any, effect upon the blood calcium. A low negative

correlation was found between the inorganic phosphorus and calcium of the blood. There was a close correlation between the phosphorus intake and the inorganic phosphorus of the blood. The question is raised: Is it possible that many cattle (even in Utah where the soil in most cases is rich in phosphorus) may lack phosphorus in their ration? It appears feasible that this could be determined by blood analyses.

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SURVIVAL OF BLISTER-RUST MYCELIUM IN WESTERN WHITE PINE [1]

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INTRODUCTION

Studies on the growth and behavior of cankers of white pine blister rust (*Cronartium ribicola* Fischer) on western white pine (*Pinus monticola* Dougl.) have been reported by the senior writer in a previous paper.[3] That paper contains data on the manner in which canker growth rates are influenced by the size and vigor of the infected stem, by regional site conditions, and by girdling and subsequent "flag formation"—a term descriptive of the conspicuous red-brown color of the foliage of the affected part after death. In that paper (p. 594) reference was made to a study of the survival of the mycelium in trunk cankers; the results of that study are presented herein.

PROCEDURE

During 1922 and 1923 the senior writer observed that blister-rust cankers on branches of western white pine in British Columbia continued to grow toward the bole of the tree after the outer portion of the branches had died and turned into "flags", and in some cases after squirrels had girdled or severed the branch at the center of the canker.[4] In order to verify the results of these observations and to determine the extent to which the rust could continue to live and grow after all source of food supply had been cut off beyond the point of infection, a number of cankered branches were so cut that part of the infected bark remained on the stub and these stubs were examined at fixed intervals. On April 25, 1924, 34 infected branches were selected on thrifty western white pines at Chee Kye,[5] British Columbia, their diameters at the base of the canker were measured, and they were then lopped off just above the lower limits of the typical orange discoloration which closely follows the progress of the mycelium in the bark of western white pine. Throughout the remainder of this paper all cankers shortened in this manner are referred to either as "cut" cankers or as "truncated" cankers. On October 25, or 6 months from the time of cutting, all the cankers were in good condition and had continued to extend toward the bole. The downward growth of the mycelium, as indicated by the downward extension of the discoloration of the infected bark, was carefully measured for each canker.

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[3] LACHMUND, H. G. GROWTH AND INJURIOUS EFFECTS OF CRONARTIUM RIBICOLA CANKERS ON PINUS MONTICOLA. Jour. Agr. Research 48: 475-503, illus. 1934.

[4] For several years numerous investigators have observed that squirrels and other rodents frequently eat the infected bark of white pine blister-rust cankers. Occasionally they completely sever the branch at the point of infection.

[5] Chee Kye is a stop on the Pacific Great Eastern Railway, approximately 40 miles, air line, north of Vancouver, British Columbia.

In April 1925 a series of 50 uncankered thrifty branches on the same area were cut in the same way that the cankers were cut the preceding year. These checks were established to determine how long uninfected branches would remain alive after cutting. In July, 42 were dead and the remaining 8 died within 2 months. During this time most of the stubs of the cankered branches cut in 1924 remained alive and the mycelium continued its growth from their cut ends toward the boles. This proved beyond a doubt that it was the presence of the mycelium in the cut cankered stubs which had kept them alive.

In the latter part of September 1925 a forest fire swept over the area and terminated the entire study before the 34 cut cankers were remeasured. Therefore growth measurements were secured for a 6-month period only—from April 25 to October 25, 1924. A comparison of the growth rate of these cut cankers with that of normal uncut cankers in the same area indicated that the downward growth rate of the cut cankers was somewhat less in each diameter class than it was for the corresponding uncut cankers. The data were too few to be conclusive; therefore plans were made for a more complete study.

A western white pine infection area near Owl Creek,[6] British Columbia, was chosen for the second experiment. Pine infection first occurred there in 1917, increased considerably in 1921, and became common in 1923 and 1924. In 1927 at the beginning of the test the white pines were young and still thrifty. They were approximately 12 to 25 years old and from 9 to 22 feet tall.

On May 6, 1927, 100 cankers on thrifty branches of 32 trees were carefully selected. All these cankers were of 1923 or 1924 origin and were therefore relatively young, most of them having produced aecia only once or twice. In general, they were one internode distant from the bole; a few, however, were almost two internodes distant. The distance from the bole to the lower limits of the cankers ranged from 2 to 17.3 inches, being more than 8 inches in only a small number of cases. The diameter of the branches at the lower limits of the cankers ranged from 0.2 to 0.9 inch. For each 0.1-inch class between these limits the number of branches was approximately the same.

Each branch was severed by a cut perpendicular to its long axis at a point about one half of an inch above the lower limit of the discoloration. On the same trees, 100 uninfected branches were cut in identical fashion to serve as checks. The check for each cut canker was on a branch of the same size and corresponding location in the tree.

In no case were any living needles or small side branches left between the cut surface and the bole; therefore it was impossible for any of the cankered stubs or checks to obtain food except from the main stem, or bole.

These truncated cankers and checks were examined in the fall of 1927 and in the spring and fall of each successive year until the spring of 1932. The condition of each was noted—whether living or dead— and, in the case of the living cankers, the downward growth from the lower limit at the time of cutting or at the time of the preceding examination was accurately measured. Of the original 100 cankers,

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[6] Owl Creek is a stop on the Pacific Great Eastern Railway, approximately 35 miles north of Chee Kye.

22 were discarded before the completion of the study because of the death of the trees on which they were located.

On May 1, 1928, a similar experiment was begun on the same area but on different trees. Fifty cankered branches and fifty uninfected checks were cut in exactly the same way as were those in the preceding year. In order to determine the immediate effect of cutting, this series was examined monthly until fall; after that the data were taken as for the 1927 series. Here again the basis for comparisons was reduced through the death of some of the trees. Data were taken on 44 cut cankers and checks in 1929 and on 32 cankers and checks in 1930. At the end of 1930 work on this series was terminated.

SURVIVAL OF TRUNCATED CANKERS

The data summarizing the condition of the cankered stubs at half-yearly and yearly intervals are shown in table 1. The condition of the cankered stubs is assumed to be the same as that of the canker itself—i.e., living or dead—for *Cronartium ribicola* is an obligate parasite and can live only so long as its host remains alive. When a canker had grown down the stub and entered the bole, the stub died in a short time. Inasmuch as the purpose of this study was to determine the effect of cutting on canker survival and growth, those cankers that died or entered the bole were not examined at subsequent dates. Because of the uniformity of the data after the 2-year period, the half-yearly intervals are not shown.

TABLE 1.—*Survival of truncated cankers and checks, Owl Creek, British Columbia*

Cankers or checks (number)	Time after cutting	Cankers			Checks dead
		Living but not yet reaching bole	Dead before reaching bole	Entered bole	
	Years	*Percent*	*Percent*	*Percent*	*Percent*
122	½	95.9	0.0	4.1	95.9
122	1	91.0	1.6	7.4	99.2
122	1½	66.4	8.2	25.4	100.0
110 [a]	2	44.6	18.2	37.3	
78 [b]	3	21.8	21.8	56.4	
78	4	5.1	29.5	65.4	
78	5	3.8	30.8	65.4	

[a] Trees containing 12 cankers died during winter.
[b] Examination of 1928 series discontinued.

Examination of the data for the 122 checks shows that 95.9 percent, or 117, were completely dead half a year after they were cut. Only 1 of the remaining 5 checks survived for 1 year, and it died shortly thereafter.

Comparison of the survival data for the truncated cankers with those for the checks clearly shows that the former remained alive for a very much longer time than the latter. This comparison is further strengthened by the results of the monthly examination of the checks in the 1928 series. Five percent of them were dead 3 months after cutting and 90 percent in 4 months, showing that the great majority of deaths occurred during the first 6 months.

In a previous reference to the present study the statement was made [7] that the survival of the cankered stubs suggested that "the presence of the infection at the end of the stub had the effect of stimulating a reversal of flow of the elaborated food materials." The data presented herein are believed to justify fully this hypothesis. The fact that with one exception the uninfected checks died within a year after cutting whereas the cankered stubs remained alive in some cases for at least 5 years shows that the presence of blister-rust infection tended to keep the stubs alive until the cankers could grow into the bole.

The action of the rust upon the stream of assimilates in the cut cankered stubs would be an interesting subject for physiological study. No other phenomena even remotely analogous to the survival of the cankered stubs are known to the writers. Certain features of resemblance are suggested by the well-known action of false-mistletoe infection, which has the effect on several hosts of prolonging the life of certain branches; notably in the case of the lower branches of *Pinus ponderosa* Dougl. and *P. contorta* Dougl. But in these cases the prolongation is attributable to a stimulation of the growth of the foliage beyond the point of infection whereby the infected portions are provided with an increased supply of elaborated food from the normal direction.

DOWNWARD GROWTH OF TRUNCATED CANKERS

As previously stated, the downward growth of all living cut cankers was carefully measured at the time of each examination. These measurements, too numerous for detailed publication, indicate three things: (1) The downward growth rate of the mycelium in the cankered stubs was constant as long as the stubs remained alive; (2) this rate was very slightly less than the downward growth rate of normal uncut and unflagged cankers; and (3) it was almost identical with the downward growth rate of flagged cankers, which is likewise slightly less than the normal growth rate. This reduced rate of growth can obtain, however, only while the fungus is in the stub or in those portions of the stem below the flag which are in low vigor because of the flagging. As soon as the infection reaches the bole or the region of the healthy living side branches below the canker, its rate of growth immediately becomes normal.

While similar in rate of downward growth, the cut and the flagged cankers are not exactly comparable as regards the length of time they may remain alive on internodes unsupported by living foliage beyond the canker. The death of the portion of the branch or stem beyond the canker in flagging reacts differently and evidently saps the vitality of the lower uninfected portion much more than when the branch or stem beyond the canker is cut off. For example, when a canker is situated so far down on a branch that most of the foliage is beyond the point of infection, the entire branch is generally killed at the time of flag formation. It has been noted, further, that flagged cankers almost invariably die out if the length of unsupported internode is so long that the canker cannot grow over it within 2 years. On the other hand, the present data show that the majority of the cut cankers

[7] LACHMUND, H. G.   See footnote 3.

remain alive for at least 2 years and that many of them survive for 3 years or more. From these observations it appears that cutting causes less of a drain on the lower stem than does flagging. Consequently the longevity and survival data for the cut cankers cannot be used as criteria for the survival of cankers after flagging.

SUMMARY

The mycelium of white pine blister rust continues to live and grow in infected western white pine bark after flagging, i.e., the death of the portion of the branch beyond the canker, has taken place or after squirrels have girdled or severed the branch at the center of the canker. In order to obtain definite information on this phenomenon the writers in the spring of 1927 and 1928 shortened 150 cankers, leaving stubs with about one half of an inch of living canker at their tips. Checks were cut in exactly the same way on uninfected branches in the same relative position in the trees.

The checks were practically all dead within 1 year from the date of cutting, whereas the cankered stubs remained alive for periods ranging up to 5 years, or until the mycelium entered the bole of the tree. The mycelium apparently stimulated a reversal of the flow of assimilates in the infected stubs.

Measurements of the downward growth of the cankers in the stub indicate that it proceeds at a constant rate slightly less than that of normal cankers but almost identical with that of flagged cankers. Flagged cankers survive for a shorter time than cut cankers.

In F_3 progenies of hybrids 43 and 44 (Markton × Iogold) there were a large number of resistant progenies in each F_2 smut group, but no progeny was classified as susceptible.

Hybrids 48 and 49 (Cornellian × Markton) involve a cross between a resistant variety and one that is rather susceptible, the smut in the Cornellian plants ranging from 27.2 to 72 percent in the different tests. As shown in table 2, a predominance of resistant progenies was obtained in the F_3. Furthermore, only four progenies were classified as susceptible, and the highest percentage of infection obtained in any of these was less than 70. The data for the F_2 and the F_3 generations do not indicate any simple dominance and segregation.

Hybrids 41 and 42 (Richland × Markton) involve a cross between a resistant variety and one that shows a very low percentage of infection. In the F_2 generation 67 plants were inoculated and only 1 was infected. Only 1 F_3 progeny was classified as susceptible, 70.5 percent of the individuals being infected. Most of the progenies were entirely resistant.

The Richland and Fulghum parents of hybrids 39 and 40 were slightly susceptible to *Ustilago avenae*-Missouri, both showing less than 5 percent of infection. In contrast to the two parental varieties, a comparatively large number of the F_2 hybrids were infected. In each of the three groups of F_3 progenies there were 2 or 3 times as many segregating progenies as there were resistant ones. Susceptible progenies also were found in each group, most of the groups containing a high proportion of infected individuals.

The number of infected F_3 progenies in each of the six crosses inoculated with *Ustilago avenae*-Missouri is shown in figure 1.

REACTION TO USTILAGO LEVIS-MISSOURI

As has already been shown, the F_2 generations of the six crosses were inoculated and tested with *Ustilago levis*-Missouri, and F_3 progenies of five of the six crosses were tested with the same race of smut. Table 2 shows that no experiments with *U. levis*-Missouri were conducted with F_3 progenies of hybrids 39 and 40 (Richland × Fulghum).

Many F_3 progenies in the *Ustilago levis*-Missouri series contained smutted individuals, although both parental varieties of the crosses were resistant (table 2). Hybrids 37 and 38 (Monarch Selection × Black Mesdag) included 128 resistant F_3 progenies, 75 segregating, and 1 that was classed as susceptible. This susceptible progeny contained 22 plants, of which 13 (59 percent) were smutted. The 75 segregating progenies contained 1,492 plants, of which 205 were smutted. Hybrids 46 and 47 (Markton × Black Mesdag) included 128 resistant progenies, 25 segregating, and none that was susceptible; the 25 segregating progenies included 493 plants, of which 50 were infected. Hybrids 48 and 49 (Cornellian × Markton) included 96 resistant progenies, 24 segregating, and none that was susceptible; the 24 segregating progenies included 479 plants, of which 43 were infected. In nearly all these segregating progenies the number of infected plants was small—usually but 1 or 2 being observed.

The number of infected F_3 progenies in each of five crosses inoculated with *Ustilago levis*-Missouri is shown in figure 2.

REACTION TO USTILAGO AVENAE-FULGHUM

Studies were made with the Fulghum race of *Ustilago avenae* only on the F_3 plants of hybrids 39 and 40 (Richland × Fulghum). In the various experiments, Fulghum showed 78.7 percent of infection with this race of smut, whereas Richland was nearly free from infection. No resistant F_3 progeny was obtained in the first group (descended from F_2 plants tested with *Ustilago avenae*-Missouri). Very few susceptible F_3 progenies were obtained in any of the groups; several might have been expected in the first and third groups, unless there was some correspondence in the inheritance of resistance to all three races of smut. Comparatively high susceptibility in the F_2 generation to all three races of smut was associated with comparatively few F_3 progenies susceptible to *U. avenae*-Fulghum.
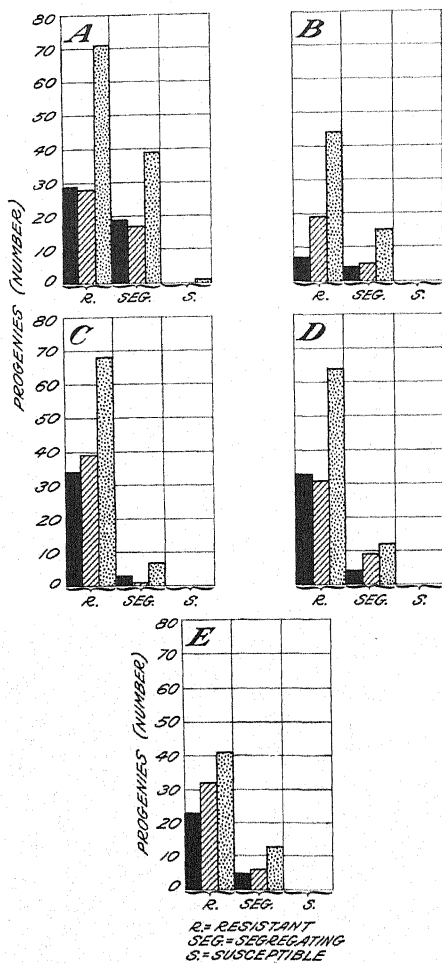
FIGURE 2.—Reaction to *Ustilago levis*-Missouri of F_3 progenies for each of five oat crosses, grown from F_2 plant populations that had been inoculated with *U. avenae*-Missouri, *U. avenae*-Fulghum, and *U. levis*-Missouri: *A*, Hybrids 37 and 38; *B*, hybrids 41 and 42; *C*, hybrids 43 and 44; *D*, hybrids 46 and 47; *E*, hybrids 48 and 49.

DISCUSSION

The results obtained from inoculating F_3 plants of hybrids 37 and 38 (Monarch Selection × Black Mesdag) with *Ustilago avenae*-Missouri show a simple dominance for resistance to this smut. These results are in accord with those reported by Reed (*8, 10, 12, 13*) in his studies on smut resistance in hybrids of Hull-less × Black Mesdag, Early Gothland × Victor, and Early Gothland × Monarch. In these crosses one parent was susceptible to the particular races of smut used and the other was resistant. The results showed a simple dominance for the inheritance of resistance, segregation occurring on the basis of a 3:1 ratio in F_2. The results obtained in F_3 were in harmony with the interpretation.

The presence of segregating progenies in F_3 in certain crosses in which no infection was expected is doubtless connected with the behavior of certain oat varieties. Occasionally an infected plant is found in a row of Markton when this variety is inoculated with *Ustilago levis*-Missouri. Fulghum frequently shows a small percentage of infection with both *U. avenae*-Missouri and *U. levis*-Missouri. Iogold and Cornellian show a comparatively high percentage of smutted plants. It has, however, been very difficult to approach 100 percent of infection in either of these varieties.

The further question arises as to the extent to which the fungus develops in individuals of such varieties as Markton, Fulghum, and Iogold, the plants of which finally appear normal. Kolk (*5*) has shown that, in the resistant Black Mesdag, *Ustilago avenae*-Missouri penetrates and ramifies more or less extensively throughout the coleoptile. Apparently, however, it does not gain entrance into the developing embryonic tissue.

Bayles and Coffman (*1*), in studies of the effect on germination of removing the hulls from seed of certain oat varieties, report a reduction of 5.1 percent in seedling emergence in the Markton variety when seed with hulls removed was inoculated with smut. A similar reduction was observed in the susceptible varieties Early Champion and Sixty-Day. Stanton et al. (*18*), reporting studies on the effect of removing the hull on seedling emergence in oat varieties, showed that although Markton gave no apparent indication of smut infection, yet seedling emergence was reduced by 2.9 percent through inoculation with smut. It was concluded from this evidence that Markton is not completely free from infection by the smut organism.

The occurrence of marked smut infection in F_3 progenies of the Markton × Black Mesdag cross is difficult to explain. It is possible that complementary factors may account for the reduction, or apparent breaking down, of resistance in the F_3 progenies of this cross. Briggs (*2*) has observed the occurrence of smutted plants in some of his resistant progenies of hybrids between Hard Federation and Hussar wheat varieties. No smut appeared in the F_2 and F_3, but in the F_4 and subsequent generations slightly susceptible families were obtained. Briggs interprets his results on the basis of modifying factors.

In the Markton × Black Mesdag cross, 25 percent of the F_3 progenies descended from F_2 plants inoculated with *Ustilago avenae*-Fulghum were infected (table 2). The occurrence of smut infection in so many progenies is hardly analogous to the appearance of a few smutted individuals in F_4 and subsequent generations of certain wheat crosses reported by Briggs (*2*). As a consequence, these results cannot be interpreted on the basis of modifying factors. It is probable that complementary factors may offer a more satisfactory basis for interpretation, since so high a percentage of the F_3 progenies were smutted. Both Markton and Black Mesdag may carry complementary factors for susceptibility, which, when brought together through hybridization, may produce smutted plants.

Another interesting result is the relation between the number of resistant, segregating, and susceptible F_3 progenies. In the *Ustilago levis*-Missouri series there is a preponderance of resistant progenies in all five hybrids. The same is true in the *U. avenae*-Missouri series of

hybrids 41 and 42, 43 and 44, 46 and 47, and 48 and 49. Of course these results would be expected to a large extent on the basis of the behavior of the parental varieties, since none of them showed anything like 100 percent of infection.

The loss of the susceptible individuals in these hybrids introduces a difficulty in the complete analysis of smut inheritance. The smutted plants being destroyed, their subsequent generations cannot be grown.

SUMMARY

This paper presents results obtained with six oat hybrids, involving crosses between varieties that differ in their behavior to certain races of the oat smuts.

F_2 plants of all these crosses, inoculated with *Ustilago arenae-*Missouri, *U. levis*-Missouri, and *U. arenae*-Fulghum, were grown and studied. Progenies of all 6 crosses inoculated with *U. arenae*-Missouri, progenies of 5 crosses inoculated with *U. levis*-Missouri, and progenies of 1 cross inoculated with *U. arenae*-Fulghum also were grown in F_3, and their reaction to these smuts was recorded.

The data for hybrids 37 and 38 (Monarch Selection × Black Mesdag) inoculated with *Ustilago arenae*-Missouri indicate that smut resistance is inherited on the basis of a 3:1 ratio.

A most noteworthy feature is the occurrence of considerable smut in some F_3 progenies of hybrids descended from entirely resistant parents.

In most of the hybrids completely resistant progenies predominated over segregating progenies; there were very few susceptible progenies recorded.

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TESTING ALFALFA FOR RESISTANCE TO BACTERIAL WILT[1]

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INTRODUCTION

In 1930 the writer (*3*)[2] described a procedure whereby it seemed possible to compare alfalfa (*Medicago sativa* L.) varieties in regard to their resistance to bacterial wilt, caused by *Phytomonas insidiosa* McCulloch, in the space of a single year, thereby avoiding the delay and climatic hazards inevitable in a field test in infested soil. The procedure described permitted not only the comparison of the degree of resistance in varieties but also the selection of resistant plants from which resistant strains may be developed. During 3 years the writer has used this method in comparing resistance in plants from a considerable number of seed lots of alfalfa. Modifications of the procedure, which would permit testing larger numbers of plants with the facilities available for the purpose at Madison, Wis., have been tested. The development of the parasitic bacteria in resistant and in susceptible plants has been compared. The present paper is a report on the progress of the work thus outlined.

In 1930 Peltier and Tysdal (*7*) published results from a single but extensive trial of the method described by the writer (*3*). In 1933 Peltier (*5*) published additional data of a similar character. All of these results are substantially in agreement with those presented here. In 1932 Peltier and Schroeder (*6*) in a study of the nature of resistance of alfalfa to wilt reached the conclusion that in the material studied resistance was in the main associated with morphological features of the root. The writer is unable to corroborate this conclusion. These results by Peltier and his associates will be discussed later in this paper.

COMPARISON OF VARIETIES FOR RESISTANCE

The procedure in comparing seed lots for resistance has been little altered from that originally suggested (*3*). The seed is planted in flats in the greenhouse, preferably in November, but when necessary as late as the middle of January. When the seedlings are about a month old they are transplanted, usually into beds where they are spaced about 1½ inches apart in rows 3 inches apart. As early in May as possible the seedlings are washed from the soil, inoculated, and transplanted to the field, where they are spaced 4 inches apart in rows 10 inches apart. In 1930, inoculation was made by scraping opposite sides of the root lightly with a knife under a bacterial suspension from several pure cultures of different origin. In the 2 following years incision was made through the bark with a sharp knife under a bacterial suspension instead of scraping. A second inoculation was

made of the plants in the entire plot in 1930 and of a part of those in the plot in 1931 in early August in the following manner. After all plants showing disease symptoms were pulled, earth was removed from around the crowns of those remaining, and a thin slice of diseased tissue from one of the infected plants was inserted in an incision through the bark of the taproot. The earth was then replaced about the crowns and the ground was well watered. This second inoculation cannot be made rapidly, and therefore it was not carried out with plants from the seed lots from Turkistan in 1931 nor was it attempted at all in 1932. Three cuttings have usually been made in the early bloom stage.

After active growth had ceased in the fall, the plants were dug for examination and divided into two classes, namely, those infected and those uninfected. Evidence of infection was first sought by cutting the main root of each plant at about 6 inches below the crown. If no discoloration characteristic of the disease could be found in any root at this distance from the crown, the scar of the wound made at inoculation was examined. If no discoloration was found here after making such incision as seemed necessary, the plant was classed as uninfected. Such an examination will not always reveal local infections near the crown, and therefore occasional plants judged uninfected and placed in the greenhouse have subsequently developed disease; but these occasional errors in classification are preferable to a more thorough examination of the plant which destroys its immediate usefulness in seed production.

The method outlined may be varied by starting the plants at different times in the year and inoculating at different stages of development in the greenhouse as well as in the field. Several variations which might be serviceable if successful have been tried. Chief among these are the following:

(1) Seed sown in the field in early spring.
    (a) Inoculation at transplanting to rows in the field, as with greenhouse-grown plants, as soon as seedlings are large enough to inoculate conveniently through wounds in the roots.
    (b) Inoculation at transplanting to the greenhouse in which the plants are grown through the summer.
    (c) Inoculation of spaced plants without transplanting.
(2) Seed sown in the field in late July or August; inoculation at transplanting to the greenhouse in October.

The first variation was tried extensively in 1930. Owing in part to a thick stand, the plants did not attain sufficient size for transplanting until late June, and transplanting extended into early July, when the temperature was high. While water was freely used at transplanting and subsequently, the plants did not make rapid growth during the entire summer, though few died. Infection was less than by the routine method, especially in the more resistant class. The period from inoculation to the end of the summer appeared much too short for advanced development of the disease in infected plants. The procedure gave useful comparisons between seed lots, but it did not result in the infection of nearly so many plants as did the routine method. It has not been tried further.

The second variation was likewise tried in 1930 in the hope of extending the growing season somewhat and thus obtaining a more thorough elimination of the susceptible plants. Seemingly excellent conditions for infection were provided by shading the greenhouse so

that the temperature did not rise above 30° C. and by keeping the soil well watered. Nevertheless, infection was even less than in plants transplanted to the field at the time the greenhouse experiment was begun. The method appeared useless and was not tried further. In general, field-grown seedlings transplanted at any other time than in late autumn make less rapid recovery and subsequent growth than greenhouse-grown seedlings.

The inoculation of spaced seedlings grown in the field by digging around the plants and applying a bacterial suspension to a wound made in the root or by inserting bits of diseased tissue in the root has invariably proved a highly effective method in securing infection. In 1931 it was tried extensively. The disadvantages of this method are the amount of labor involved and the fact that in this latitude seedlings from the spring sowing do not attain sufficient size for inoculation until the growing season is far advanced. Thus when winter comes the disease has not progressed far, especially in the more resistant plants. It should be noted that inoculation by fragments of diseased tissue dipped in bacterial suspension and inserted in slits in the bark of the roots has given slightly higher infection than any other method tried. Tables of the results of this method as compared with results obtained by the use of several pure cultures are omitted for the sake of brevity.

Where seedlings grown in the field in late summer and early autumn were inoculated upon being transplanted to the greenhouse, it seemed that inoculation would be made into the most susceptible tissue the plant would produce under field conditions and that in consequence disease development would be rapid. In a single trial made with about 900 plants from 12 seed samples the anticipated high infection was obtained, an infection approximately equal to that obtained in the field from the inoculation of greenhouse seedlings. The method has not been used further because it requires much greenhouse space.

TABLE 1.—*Comparative test of alfalfa varieties and seed lots for resistance to bacterial wilt, 1930*

[Inoculation was made with pure culture at transplanting in May and by use of diseased tissue in August]

Origin or variety	Seed lot no.[a]	Plants inoculated		Origin or variety	Seed lot no.[a]	Plants inoculated	
		Total	Unin-fected			Total	Unin-fected
		Number	*Percent*			*Number*	*Percent*
Arizona	1289	217	1.5	Montana	1300	218	2
Argentine	1332	201	1.5	Peruvian	1290	219	0
Baltic	597	181	1	Ladak	1334	233	30
Cossack	1260	145	1.6	South Dakota	875	216	4
Grimm	999	437	1	Do	1173	228	2
Do	1335	195	1	Turkistan	1356	149	24
Kansas (Wichita)	1224	302	0	Do	⌠ 84371	118	44
Kansas (Derby)	1225	209	9.5		⌡ b P.I.		
Do	1301	166	5	Utah	1157	158	3
Do	1302	194	1	Do	1219	134	0
Do	1303	202	2.5	Do	1245	225	.5
Do	1304	253	3				

[a] Identification number used at the Wisconsin Experiment Station.
[b] Identification number of the Division of Plant Exploration and Introduction, Bureau of Plant Industry, U.S. Department of Agriculture.

From this survey of possible variations in the methods for determining resistance in 1 year, it appears that the schedule originally suggested best combines convenience and effectiveness. The first extended comparison of seed lots of alfalfa by this routine method for determining wilt resistance was made in 1930. The seed samples used were largely American regional strains contributed by Prof. L. F. Graber, of the Wisconsin Agricultural Experiment Station. The results of the 1930 trials are presented in table 1. Very few plants from the American strains remained uninfected, whereas the two seed samples from Turkistan and the one from Ladak were strikingly distinguished by the comparatively large number of uninfected plants.

In 1931 some of the seed samples collected by H. L. Westover in Turkistan in 1929 and in Spain and northern Africa in 1930, together with a few miscellaneous samples of diverse origin, were tested. The plant populations from seed of undoubted Turkistan origin were sharply distinguished from all others by greater resistance. The results are summarized in table 2. A few additional samples from the same regions were compared in 1932 with the same result. This outcome appears to accord with the very extensive results obtained by Peltier and Tysdal (7) and by Peltier (5). The percentage of uninfected plants in the results cited is usually higher than that shown here. Such difference in the susceptible varieties may be due to the fact that in this class of plants a second inoculation was made by the writer, whereas in obtaining the results cited, but a single inoculation was made.

TABLE 2.—*Resistance to bacterial wilt in alfalfa from foreign sources after artificial inoculations in 1931–32*

Source	Seed lots	Plants inoculated			
		Number	Percentage uninfected		
			Highest	Lowest	Average
Turkistan:	*Number*		*Percent*	*Percent*	*Percent*
Samarkand	4	577	80	33	55
Tashkent	11	1,926	80	23	50
Ferghana	2	291	52	43	47
Khiva	1	59			54
Bukhara	1	108			69
Manchuria	1	63			0
China	4	390			.3
Spain	45	4,873	1	0	.3
Portugal	4	500	1	0	.2
Northern Africa	33	4,203	1	0	.5
South America	4	491			.2

Alfalfa, then, falls into two distinct major classes with respect to resistance: A resistant class consisting of or derived more or less directly from Turkistan or Ladak, and a susceptible class comprising alfalfa from all other sources tested thus far. The result may be stated in another way. Resistance to bacterial wilt appears to be a characteristic of alfalfa in the region, or at least in much of the region, credited as being its place of origin. The boundaries of the region in which high resistance is a uniform characteristic are not precisely delimited by the data at hand. In the dissemination of the plant from this region of origin and adaptation to other regions

where it is now grown the resistant character appears to have been largely lost, whatever habit of growth the plant has assumed in its new environment. That the disease occurs in Turkistan is attested by the fact noted previously,[3] that the bacteria causing it were recovered from two plants collected by Westover at a small village north of Bukhara. However, it is unlikely that the disease is abundant or conspicuous in this region at present, as Westover has stated to the writer that he recognized the disease only at this location, in a field that had been excessively watered. Thus it appears possible that the parasite has been constantly associated with the plant in Turkistan, enforcing a selection for resistance in that region, and that the parasite has not followed the plant elsewhere to continue that selection until a comparatively recent introduction into the United States; but facts are not at hand whereby this suggestion may be confirmed.

COMPARISON OF INDIVIDUAL PLANTS FOR RESISTANCE

Whatever the historical background of the present regional separation of the resistant and susceptible varieties of alfalfa, the present task is production by artificial selection of resistant varieties suited to regional needs. Two sources of material are available for use: (1) Strains of highly resistant alfalfa from central Asia and (2) individual resistant plants that may be found in present commercial varieties. If individual plants from susceptible varieties are chosen for the building of varieties, much use of artificial inoculation for the elimination of susceptible plants from populations will be required. Therefore, a critical appraisal of the accuracy and limitations of artificial inoculation as a tool both in comparison of varieties and in selection should be made.

That a test carried out in the field where conditions differ from year to year will give identical results is hardly to be expected; and in fact it has not always given closely similar results in the same season from transplantings made but a short space of time apart. Large lots of seedlings have been divided and transplanted at several dates through May and June, but differences in the results of these experiments have not been greater than differences that have occurred from inoculations made a few days apart. In a few Turkistan seed samples differences in the percentages of uninfected plants have amounted to 30 percent in exceptional cases. In susceptible varieties differences have not been so great, due perhaps in part to the second inoculation. Such differences were not only perplexing in the plots at Madison, but became even more so when an attempt was made to duplicate some of the work elsewhere. For instance, in 1930 some of the seed samples numbered in table 1 were grown by C. O. Grandfield at Manhattan, Kans., in the greenhouse, and inoculated at transplanting with the same cultures as at Madison. At Manhattan the percentage of uninfected plants was determined by Grandfield in the autumn. On the whole, the percentage of uninfected plants was greater at Manhattan, probably because they were inoculated but once, but this result was not consistent in all the lots. For instance, one sample gave 25 percent of uninfected plants at Manhattan and but 2.5

[3] JONES, F. R. BACTERIAL WILT OF ALFALFA IN TURKISTAN. U.S. Dept. Agr. Bur. Plant Indus. Plant Disease Rptr. 14: 125. 1930. [Mimeographed.]

percent at Madison, and a sample giving 7 percent of uninfected plants at Madison gave but 2.5 percent at Manhattan.

A study of planting and climatic data has been made in an effort to explain these differences; but thus far no fact or set of facts has been found with which the differences can be correlated. That the method of inoculation is usually sufficiently effective to prevent many plants from escaping infection, even though they escape wounding, was shown in 1931, when groups of 10 plants were set unwounded and uninoculated at intervals in the plot so that difference in growth between diseased and healthy plants might be observed. At the end of the season all these groups of uninoculated plants were found infected almost as severely as the inoculated plants, except in the final transplanting made early in June. Emphasis upon these irregularities in the results of inoculation should not obscure the fact that most repeated trials are closely consistent in result. A second inoculation of plants that have not shown evidence of disease in the foliage by the end of July appears to make results more consistent than does a single inoculation. However, this biological test, carried out in the field under varying climatic conditions, has not been and perhaps cannot be standardized to produce precise comparisons.

Thus far the discussion has been confined to the consistency of the test in defining the resistance of plant populations. In the selection of resistant plants it is even more important to know how accurately the test defines or can be adapted to define the resistance of individual plants. At the conclusion of a test in autumn some plants are found uninfected, others with varying degrees of disease development. The questions arise whether any or all of these uninfected plants are uninfectable, or immune, and whether degrees of disease development represent intrinsic resistance or a more or less accidental retardation in disease development. In an effort to answer these questions, plants showing apparent degrees of disease development were set in the greenhouse for further observation. Most of these plants developed disease and soon died. Thus the tentative conclusion was reached that degree of infection in plants from susceptible varieties, even after two inoculations, is not a reliable index of degree of resistance in those plants, at least, not of a sufficiently high degree of resistance to be of practical importance. In fact, occasional plants among those showing no infection at all have developed disease and died as though very susceptible. These results may indicate that resistance, at least a low degree of resistance, is not stable in growing plants. Thus one of the first and most essential steps in the study of resistance in individual plants consists in the determination of the stability of resistance with reference to the age and environment of the plant, or the description of such degrees of resistance as may be distinguished.

A simple procedure in the determination of the stability of high resistance in plants with reference to age consists in the repeated inoculation of such plants as fail to be infected by the routine method used in the comparison of plants. A considerable number of plants found uninfected in the fall have been reinoculated and set in the ground for growth the following year. In spite of winter protection, such transplanted roots have been killed outright or so badly damaged that no significant results have been obtained. Inoculated populations left in the ground all winter with some protection have been dug

in the spring, and the uninfected plants have been reset in the ground. These have usually failed to grow vigorously after such transplanting; consequently, the reset plants can hardly be regarded as representing the condition of plants that have grown with undisturbed roots. A more promising method of obtaining the desired end consists in the use of cuttings. In the autumn of 1930, cuttings were made from a number of uninfected plants and rooted in the greenhouse. The vigorous plants from these cuttings were inoculated by inserting bits of diseased tissue in the roots and were set in the field in the following spring. A part of the result of this inoculation is given in table 3. Some of the groups of genetically identical plants obtained from cuttings remained free from infection and some groups were infected in part or in all of the individuals. Further trial will be made of these uninfected populations to determine whether infection can be obtained in them; but at present it appears that even from varieties in which 95 percent of the plants are infected in a routine test individuals may be found which are immune to wilt under these summer field trial conditions.

TABLE 3.—*Infection of plants from cuttings the parent plants of which had withstood two inoculations without infection*

Variety	Plant no.	Diseased	Healthy	Variety	Plant no.	Diseased	Healthy
		Number	*Number*			*Number*	*Number*
	480–1	3		Montana Common	1300–1		4
	480–2		7		1300–3		2
	480–6		4		1303–2		3
	480–7	4			1303–3		3
Turkistan	480–9		5		1304–1	4	3
	480–10	2	2	Kansas Common	1304–2	1	2
	2–5		11		1304–4	1	4
	2–7	4	3		1304–5		2
	1356–5	2	5		1304–7	5	
Grimm	998–1	4			1304–8	2	2
	1210–1	1	1				
Utah Common	1158–3	4	1				
	1158–4	2	1				

Infection that occurred in this trial was usually slight. No symptoms were observed in the foliage. The disease had progressed very slowly after entering the vascular system. Although the fate of these infected plants in the following year was not determined, it seems probable that in most cases the disease would have been outgrown. On the basis of this opinion, such plants may be placed in a highly resistant class in which infection is possible but in which it tends to remain localized.

A tentative third class of plants, having resistance slightly lower than the preceding yet perhaps enough to be of practical importance if possessed uniformly in a variety, is illustrated by the following example: Three plants of Grimm, two without infection, the third with very slight infection, were selected at the end of the inoculation trial in 1930. Cuttings from these plants were grown in the greenhouse and three from each plant were inoculated in the spring of 1931. At the end of the summer none of these plants showed disease, and they remained in the field through 1932, still vigorous and without evidence of disease in the foliage. When they were dug in the winter, they were found badly infected, invasion extending to the center of the root, and they soon died. Degree of infection and behavior differed little in the nine plants. They were much less

injured by wilt in 2 years than most Grimm plants in a single summer; therefore, they may be regarded as having a degree of resistance somewhat lower than that described in the previous class.

Thus, by means of these field trials of cuttings from individuals, resistant plants may be classified provisionally as immune, highly resistant, and resistant. Such classification is admittedly very crude and unsatisfactory. It may serve to indicate that resistance appears to exist in varying degrees in plants. In rare individuals immunity may be attained, but comparatively high and low degrees of resistance may be distinguished.

THE NATURE OF RESISTANCE

During the foregoing comparison of varieties with respect to resistance, detailed study has been attempted of the behavior of infection in plants having different degrees of resistance, with a view to describing the differing host-parasite relationship which finds conspicuous expression in these degrees of resistance. In this work methods of approach have been developed and tentative conclusions have been reached which need further confirmation. A discussion of this incomplete work seems warranted, since Peltier and Schroeder (6) in a recent publication have reached conclusions radically different from those of the writer.

In the course of the comparative testing of alfalfa seed lots many inoculated plants of resistant or susceptible strains in various stages of disease have been examined and compared. Material has been fixed and stained after being embedded in paraffin, but more frequently living roots have been sectioned with the razor or the sliding microtome. By the latter method discolored areas indicating infection can be examined at the exact point of interest more rapidly and effectively than is possible after the material has been embedded. Sections from living material are fixed in alcohol to hold the bacteria in place and are stained in the usual manner. Thus the relation of the bacteria to large areas of the host tissue may be surveyed in a relatively short time.

Attention was first directed to the initial step in infection, namely, the development of the bacteria in parenchymatous tissue around the wound to which the bacteria were applied. Here it was found that in plants with no vascular invasion at the end of a routine test for resistance, there is usually no invasion of parenchymatous tissue or but a very slight invasion which has not reached the vessels. In plants in which vascular invasion is slight, invasion at the point of inoculation is slight and is not found elsewhere along the invaded vessels. In very susceptible plants invasion of parenchymatous tissue at the point of inoculation is relatively rapid and abundant, and vascular invasion is soon followed by parenchymatous invasion at more or less widely scattered points along the invaded vessels. Occasionally very conspicuous exceptions occur in which extensive invasion of parenchymatous tissue at the point of inoculation is not followed by extensive vascular invasion, but these exceptions appear to form a very small distinct class which need not confuse the discussion here.[4]

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[4] In the exceptional plants referred to, the parenchymatous tissue appears to react to invasion by hypertrophy and even by slight hyperplasia. The region of inoculation develops a gall-like swelling. The bacteria usually pass to the very center of the root along the enlarged wood ray cells. Few vessels are nvaded, and these may be scattered to the center of the root. Such plants have been found thus far only n strains of Turkistan alfalfa.

The foregoing results indicate that resistance first finds expression in the failure of the bacteria to establish themselves rapidly and extensively in the parenchymatous tissue of the host, either at the portal of entry, or later from the invaded vessels.

Following the examination of infection in populations presumably more or less heterogeneous, similar comparisons were made with plants of known uniformity, that is, with cuttings from previously tested plants. The supply of such plants has been small, both in quantity and in diversity of origin; therefore, methods were devised whereby it could be used most economically. Chief among these were the inoculation of pieces of roots in an incubator and the reciprocal grafting of plants. The results of the preliminary tests are as follows.

Pieces of roots taken from large plants in the fall were stored under controlled environmental conditions, and infection has been obtained in such pieces as well as in entire plants. The procedure has been used both to test rate of penetration of the bacteria in susceptible plants under different environmental conditions and to compare penetration in susceptible and resistant roots. Results obtained in this way support observations made in growing plants. Very little penetration of the bacteria into the phloem of roots of well-attested resistance has occurred, while abundant penetration of bacteria into roots of susceptible plants has been obtained.

The most spectacular contrast in the development of the bacteria in resistant and susceptible plant tissue has come from inoculation of grafted plants, though this work is as yet meager in quantity. The grafting is easily accomplished between roots of the same diameter, both in the fall with plants from the field and in the spring with greenhouse seedlings ready to set in the field. The usual whip graft has been used, the union tightly bound with twine and covered with melted beeswax. The grafts are packed in moist sphagnum, with a little of the crown exposed, and maintained at a temperature of about 25° C. in a moist chamber for 2 or 3 weeks to hasten the union. The plants are then set in soil with protection from drying until vigorous growth starts. Inoculation has occasionally been made on the cut surfaces at the time of grafting, but usually in the susceptible portion of the graft at the time of planting.

The two portions of the graft appear to maintain essentially their original susceptible or resistant characteristics in this union, although a susceptible top on a resistant root lives longer after infection than it does on its own decaying root. Through the use of grafted plants bacteria can be introduced directly into unwounded and continuously functioning vessels of resistant tissue without having to pass through parenchymatous tissue in which, as previous observation indicated, they were hardly able to penetrate.

A typical contrast of bacterial development in tissues of immune root inoculated in this way with that in a susceptible root is furnished by a fortunate graft in which about 4 cm of immune Turkistan root was inserted in the taproot of a highly susceptible nonhardy plant. This graft was made in December 1931. The lower portion of the susceptible root was inoculated, and the plant grew slowly in the greenhouse during the winter. In the spring it was set in the field, where it grew vigorously, showing no symptoms of disease. When dug in October the taproot was about 2 cm in diameter and the graft

unions were smooth, though visible. The crown and taproot were then split vertically; one half was waxed over the wound and set in the greenhouse, and the other half was prepared for microscopic examination. On December 19 the foliage of the greenhouse plant showed symptoms of wilt and was then examined. Sections prepared at the earlier and later dates from this plant show differences only in degree of disease. The entire root, including the inserted portion, was deeply discolored. The new growth formed since the union was a wide band each of summer and autumn wood sharply distinguished. The lower susceptible portion of the root was severely diseased, with extensive invasion of the fall wood both in vessels and in parenchymatous tissue. The upper susceptible portion was less severely invaded, although bacteria had advanced to some of the outermost vessels. In the immune insert, which was almost as deeply discolored as the rest, the bacteria were found only in a few vessels of the summer wood and not anywhere in parenchymatous tissue. Extensive gum formation occurred in the susceptible portion, but only in the summer wood of the immune insert. The discoloration of the immune portion was due largely to the soluble stain, which may have been produced in the susceptible part. So far as could be discovered, vascular connection through the inserted portion was open, permitting distribution of bacteria through it from the infested vessels at either end, and perhaps the bacteria found there had arrived by this route. Previous trials with ink in grafts had demonstrated open vessels through the unions permitting ink particles to pass, though many vessels had sharp turns conducive to clogging.

In other inoculated grafts of resistant tops on susceptible roots, and vice versa, made at about the same time, similar results have been obtained. Disease has progressed in the usual manner in the susceptible portion. Discoloration extends far into the resistant part of the union, although few vessels in that resistant part show clear evidence of bacterial development in those vessels by the presence of matlike colonies over pits. Thus it appears that immunity and very high resistance are manifest not only by failure of the bacteria to develop in the intercellular spaces of the parenchyma of an inoculated plant, but also by a much less luxuriant growth in the pits of those vessels. In no case have bacteria been found invading parenchymatous tissue in the highly resistant part of grafts, however extensively this has occurred in the susceptible part.

From the writer's previous studies of this disease the conclusion has been drawn that without invasion of parenchymatous tissue vascular invasion does not proceed far; and thus if this apparent resistance of the parenchymatous tissue to invasion continues to be found in all plants which are not infected or but slightly infected by repeated inoculation in the usual routine, the resistant character seems to be manifest chiefly in the parenchymatous phase of invasion. The nature of this resistant character is not apparent. The gathering, testing, and increasing of resistant plant material for comparison is in progress, and a more comprehensive comparison of such material is planned.

A theory of the origin of resistance very different from that just outlined has been stated recently by Peltier and Schroeder (*6, p. 2*).

* * * The usual or normal progress of the bacteria from their entry until the death of the plant ensues has been followed.

In the main it has been found that resistance in some alfalfas is associated with certain morphological features, particularly in the root, which inhibit rapid development and invasion of the vital tissues by the bacteria.   These morphological differences in susceptible and resistant sorts are inherent, though not absolute, since any variety or strain of alfalfa is made up of a widely diverse lot of individuals.   It is for this reason that not a single variety or strain of alfalfa has been found which is completely resistant.

* * * Thus while resistance in alfalfas to wilt is associated with root structure, it is also true that inhibiting or accelerating the rate of growth of either susceptible or resistant sorts will modify the root structure to such an extent that susceptible sorts will become more resistant or resistant alfalfas more susceptible.

* * * There appears to be no direct evidence in any of our physiological or microchemical studies to show that any internal physiologic function of the plant makes one variety more resistant than another, except insofar as morphological modifications may occur under different environmental conditions.

The morphological characteristics of resistant varieties are stated to be as follows: (1) Vessels of smaller diameter, more angular, with heavier wall thickenings; (2) vessel segments shorter, with more obstructions from remaining vestiges of the septa; (3) vessels arranged in groups, with less contact and more intervening fibers; and (4) less frequent contact of vessels with parenchymatous cells. Peltier and Schroeder illustrate these differences by contrasting photomicrographs, but give no measurements of length or diameter of vessel segments. Measurements made by the writer from their photomicrographs (*6, pl. 7*) showing difference in diameter of vessels in a plant of a resistant and a susceptible variety show that the average outside diameter of vessels in the resistant root is about 23μ and in the susceptible root about 38μ. The writer has examined sections of many resistant and susceptible plants and has not observed such contrast in vessel diameter. However, in such comparisons, measurements are a safer guide than observation, and therefore, in July 1933, plants of 3 resistant and 4 susceptible varieties under test in the field were sectioned for examination. Median longitudinal sections were stained lightly with thionine, and the length and diameter of vessel segments of at least 10 representative vessels distributed across the diameter of the root were measured. The average of these measurements was found, and finally the average of such measurements from 5 plants. The results are given in table 4. No significant difference appears in either length or diameter of vessel segments between the resistant and the susceptible varieties. In all plants vessel diameter is approximately the same as that shown in the cited illustrations of a susceptible plant (*6, pl. 7, b*).

TABLE 4.—*Comparison of average length and diameter of vessel segments in roots of resistant and susceptible varieties of alfalfa under test for resistance to wilt*

[Measurements made in plants taken from the field, July 19, 1933]

Resistant variety	Average length	Average diameter	Susceptible variety	Average length	Average diameter
	μ	μ		μ	μ
Turkistan	104	32	Grimm	84	38
Hardistan	90	38	Cossack	80	38
Ladak	86	36	Canadian Variegated	88	36
			South Dakota Common	80	38

The writer has sought among the plants in his selections for evidence of the remaining differences between resistant and susceptible plants described by Peltier and Schroeder and has failed to find any of them. It will be very interesting indeed if these investigators have found an environmental condition in which resistant and susceptible varieties show different internal structures, but such structural differences can hardly be regarded as a cause of resistance when similar contrasts in resistance are shown by the same varieties grown in an environment in which these structural differences do not occur. The structural differences between roots of resistant and susceptible varieties here described appear closely similar to those observed by the writer in plants responding to a short period of illumination with short internodes and with long internodes, respectively, when both are grown in short days.

It should be pointed out that Peltier and Schroeder appear to the writer to have built their morphological theory of resistance upon a conception of the development of the disease in the plant which differs in one important respect from that held by the writer (*2, 4*). The difference in opinion concerns the route by which the bacteria spread within the plant, and may be stated thus. Both agree that in entering a plant through a wound the bacteria first establish themselves in parenchymatous tissue, whence they pass into vessels. From this point interpretations differ. The writer finds parenchymatous invasion arising along those vessels first invaded and believes that by passing between cells of this tissue the bacteria invade other vessels in the same manner in which the first vessels were penetrated at infection. Peltier and Schroeder do not describe such parenchymatous invasion, progressively increasing from initial infection, but describe the passage of bacteria from vessel to vessel by penetration of the thin wall separating opposed pits of contiguous vessels. Although the writer has recognized that passage of bacteria from vessel to vessel does occasionally take place by the dissolution of cell walls (*4, p. 70, fig. 18*), he has been unable to find convincing evidence that passage occurs through pores in pits as described by Peltier and Schroeder (*6, pp. 7–8*). In fact, the writer is unable to verify the existence of the pores described in the cell membrane. The photomicrographs cited as showing these pores or reticulated condition of the membrane (*6, pl. 1, B, C.*) appear to show only a refractive effect of the lignified thickening of the wall, not pores in which bacteria can or do find lodgment and development. In a recent paper, Bailey (*1*) gives illustrations and a description of vestured pits that appear to identify as vestured pits the pit condition in alfalfa illustrated by Peltier and Schroeder.

It appears probable to the writer that Peltier and Schroeder overlooked the presence of parenchymatous invasion in the early stages of disease development, widely scattered and tenuous as it often is along the invaded vessels. For instance, they state (*6, p. 27*):

As a rule plants in which bacteria are present in the parenchymatous tissues in the fall do not survive the winter or spring because of the small amount of root reserves found in plants in this stage of disease development.

This statement does not accord with the writer's experience. In the spring of 1933 special search was made for local invasions of parenchyma in plants overwintering with but a trace of disease. Among plants brought from an old field, invasions in parenchyma

were demonstrated by staining in 80 percent of the plants examined, and in 90 percent of overwintering plants artificially inoculated the previous summer. Invasion of parenchyma has been found, in the writer's opinion, sufficiently associated with all stages of active disease development to account for progress of bacteria to new vessels.

RESISTANCE AS INFLUENCED BY SELECTION

A single experiment indicating the increase in resistance that may be obtained from selection alone will be reported here. In the summer of 1931 a small field planting of cuttings of highly resistant plants, well separated from any susceptible plants, set seed. The planting consisted largely of selections from susceptible common alfalfa, with a few plants of Ladak and of Turkistan origin. The open-pollinated seed from each individual plant represented was kept separate and plants from this seed were tested for resistance the following year, in some cases in comparison with a part of the original seed lots. The results of this test are summarized in table 5. In the 1930 test of the seed lots the plants were inoculated twice; in the 1932 retrial of those lots and in the test of the seed from the selections the plants were inoculated but once. The percentage of resistance given for the selections is an average of several trials in the same plot, the number of plants included ranging from 60 to 370. Differences between resistance in parent stock and selections in the first three instances and in Ladak do not, in the writer's opinion, represent a significant increase in resistance in the selection; but in all other cases the increase is regarded as highly significant. This result indicates that considerable increase in resistance can be obtained from some of the resistant plants selected by the procedure here described. Moreover, the resistant selections in this test appeared to have the general character of the stock from which they came and were not of the early-dormant Turkistan type. Additional selections from this plant material and from other sources are being made for a further test of the possibility of increasing resistance in strains from selected plants.

TABLE 5.—*Comparison of resistance in some highly susceptible seed lots with resistance in the F_1 population of selected resistant plants from those seed lots*

[Resistance is represented by the percentage of plants remaining uninfected at the end of a routine inoculation trial]

Parent stock no.	Variety	Resistance of parent stock		Resistance in F_1 population from numbered selected plants (1932) [b] [c]				
		1930 [a]	1932 [b]	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
1210	Utah Common	0.5		11.7	8.7			
1225	Kansas Common	.5	14	15				
1245	Utah Common	1	22	22				
1289	Arizona Common	1.4	3.4	23.5	16			
1300	Montana Common	1.4	3.4	25	16			
1301	Kansas Common	2.4		40	23.3			
1302	Do	1.8	7	49				
1303	Do	32.4		35.3	38.7	37.5	66.6	
1304	Do	2.8	7	26.6	33.1	31	29.8	40
1334	Ladak	30		36				
1335	Grimm	1		31				
1356	Turkistan	24		66	43.7	59.3		

[a] Inoculated twice.   [b] Inoculated once.   [c] Average of several trials.

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SUMMARY

Alfalfa plants grown from seed from various sources have been inoculated at Madison, Wis., during 3 years for two purposes, namely, the comparison of resistance in the sources represented and the selection of resistant plants from which resistant strains may be developed.

Several procedures by which resistance in plants from different seed lots may be compared are described and evaluated.

Alfalfa from Turkistan and Ladak shows far more resistance than that from any other source from which seed has been tested thus far. However, occasional highly resistant plants are found from other sources.

On the basis of the routine inoculation used in the tests, resistant plants have been tentatively grouped in three classes, namely, immune, highly resistant, and resistant.

Resistance appears to be exhibited largely in the parenchymatous tissue of the plant through which the bacteria make little or no progress in resistant plants, and somewhat through the less rapid multiplication of the bacteria in the vessels.

No evidence has been found of morphological differences distinguishing resistant plants.

Increase in resistance in open-pollinated progeny from some of the selected plants has been obtained.

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A GALL SIMILAR TO CROWN GALL, PRODUCED ON GYPSOPHILA BY A NEW BACTERIUM [1]

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INTRODUCTION

Galls on the crown and roots of *Gypsophila paniculata* L. were first brought to the attention of pathologists of the Bureau of Plant Industry in the summer of 1932, when a grower submitted specimens of galls that had occurred in an ornamental-plant nursery in the eastern part of the United States. Galls on *Gypsophila* apparently were seen for the first time in 1931 by eastern growers of this important ornamental plant. However, neither the damage to plants nor the financial loss was extensive; consequently the growers did not at that time bring the disease to the attention of Federal workers interested in plant-disease problems.

In 1932, of 12 nurserymen who produced *Gypsophila paniculata* for the wholesale trade, only 2 were familiar with the disease, and one of these minimized his losses. He admitted that he understood the potential danger but stated that his loss had been less than 1 percent. The losses of the nurseryman who submitted the galled plants in 1932 amounted to about 25 percent.

A note regarding the discovery of the disease was published in the autumn of 1932.[2]

THE DISEASE

The galls occur principally on grafted plants in the region of the graft. They are of a soft nodular type, ½ to 3 cm in diameter (fig. 1, *A*), and may extend around the greater part of the stem or root, eventually causing the death of the plant. It is the practice of the eastern *Gypsophila* growers to lift seedling plants in the fall to use later for grafting with the desired variety. Should their field plants be galled in the summer, they are worthless when dug.

Crown gall of ornamental plants, of vegetables, and of fruit trees, which is produced by *Bacterium tumefaciens* Smith and Town., is so well known and wide-spread that no surprise is manifested when a new host plant for the disease is found. Consequently, when the *Gypsophila* gall was received and examined and its outward appearance was observed to be very like crown gall, it was at first thought that *Gypsophila* was a new host for crown gall and that the disease was not new.

The routine work for identification, however, changed this idea. Cross sections examined under the microscope showed water-soaked areas and masses of bacteria streaming from the tissues, characters that are unlike crown gall. No nematodes or fungi were present. The disease was not crown gall, but there was a possibility that it might be related to one of the other bacterial plant tumors, namely,

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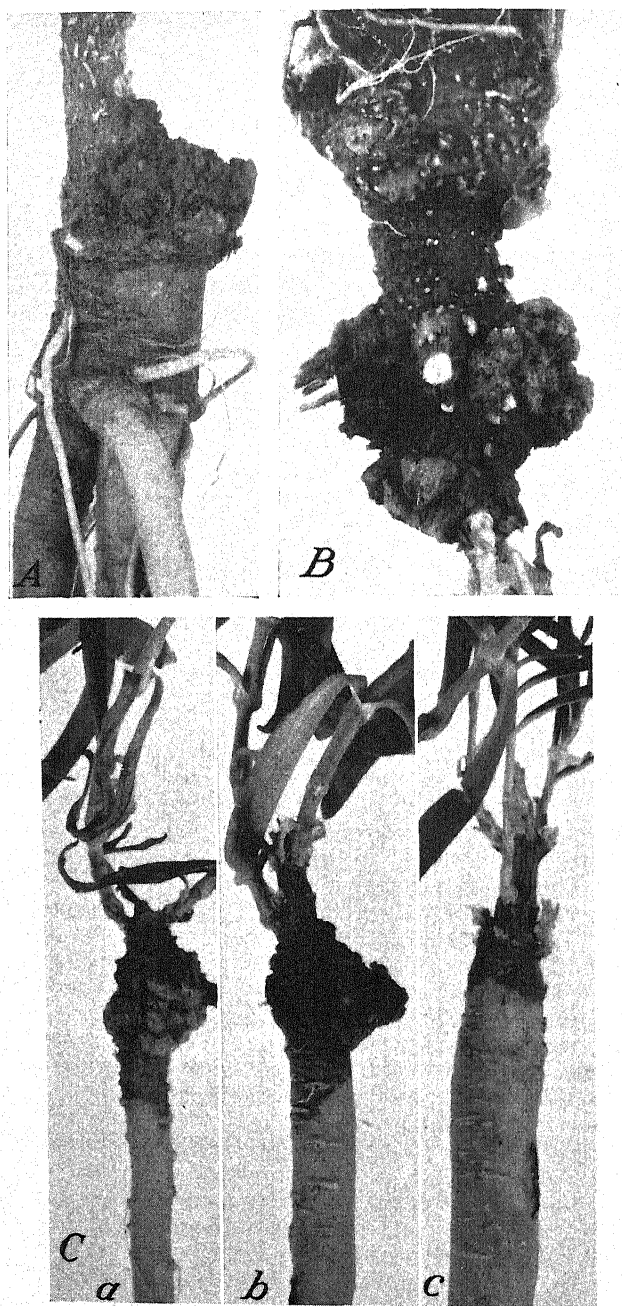
FIGURE 1.—*A, Gypsophila paniculata* gall from an Eastern State. *B, G. paniculata* gall 5 weeks old, produced by inoculating with an organism isolated from *A.*   *C,* Seedling *G. paniculata* plants; *a* and *b,* Inoculated with 2 different colonies reisolated from gall *B,* which produced these galls in less than 1 month; *c,* control plant. Natural size.

the pocket disease of the sugar beet, produced by *Bacterium beticola* (Smith, Brown, and Townsend) Potebnia; the olive knot, produced by *Bact. savastanoi* E. F. Smith; the oleander gall, produced by *Bact. savastanoi* var. *nerii* C. O. Smith; or a canker of ash trees, produced by *Bact. savastanoi* var. *fraxini* Brown.

A further study of the structure of the *Gypsophila* gall, however, showed that it had features unlike these last-named tumors and could not be classed with them. There were no gum pockets in any of the *Gypsophila* galls received for examination. There were a few brown areas in some of them, but whether the discoloration was the beginning of the break-down of the gall tissue due to the invading organism or to a reaction of the gall tissue to the byproducts of this organism is not known. No browning occurred in the galls produced by inoculation. The *Gypsophila* galls are so soft that disintegration occurs very easily.

The galls were studied in cross section (fig. 2) and in the water-soaked areas motile bacteria were seen in the cells. Some of the cells were filled with them, others were partly filled, while many had none. A few of the tumor cells were packed with crystals. The cells near the periphery of the galls contained the greatest number of bacteria. The causal organism of crown gall has not been seen in the natural gall.

The structure of the *Gypsophila* galls was found to be much like that of crown gall. Nests of rapidly developing cells could be distinguished in which parenchyma and sclerenchyma cells were mixed irregularly (figs. 2, *A*; 3, *C*).

ISOLATIONS AND INOCULATIONS

COLONIES

An organism was isolated from several galls, the same type of colony appearing on the plates poured from each gall. The colonies were abundant and apparently consisted of the pathogene responsible for the disease. They appeared in 24 hours, were translucent white in reflected light, circular, 2 to 4 mm in diameter, slightly raised in the center, and finely granular (fig. 2, *B*). Buried colonies were mostly lens-shaped, but some were round. There were no irregular colonies. In 3 days they were a creamy yellow, 4 to 7 mm in diameter, and on thinly sown plates, 8 to 11 mm. Both rough and smooth colonies appeared on the plates isolated from the same gall, but the smooth colonies predominated. Both types of colonies were used for inoculations and produced galls of similar size, although the rough type produced them a little more slowly than did the smooth type.

By means of needle pricks grafted *Gypsophila* plants were inoculated at the crown and also on the stems with several of the isolated colonies. There was a beginning of gall formation at the crown in 7 to 9 days. In 2 weeks light-colored nodular galls 1 to 1½ cm in diameter showed at the crowns. Some of the inoculated plants were slow in showing infection, but in 3 weeks all had definite galls (fig. 1, *B*). When they were 2½ to 3 cm across, quite frequently disintegration began (fig. 4, *A*). Cankers, instead of galls, were produced on the inoculated aerial stems. On the 24 plants inoculated, the infection on crown and stem was 100 percent.
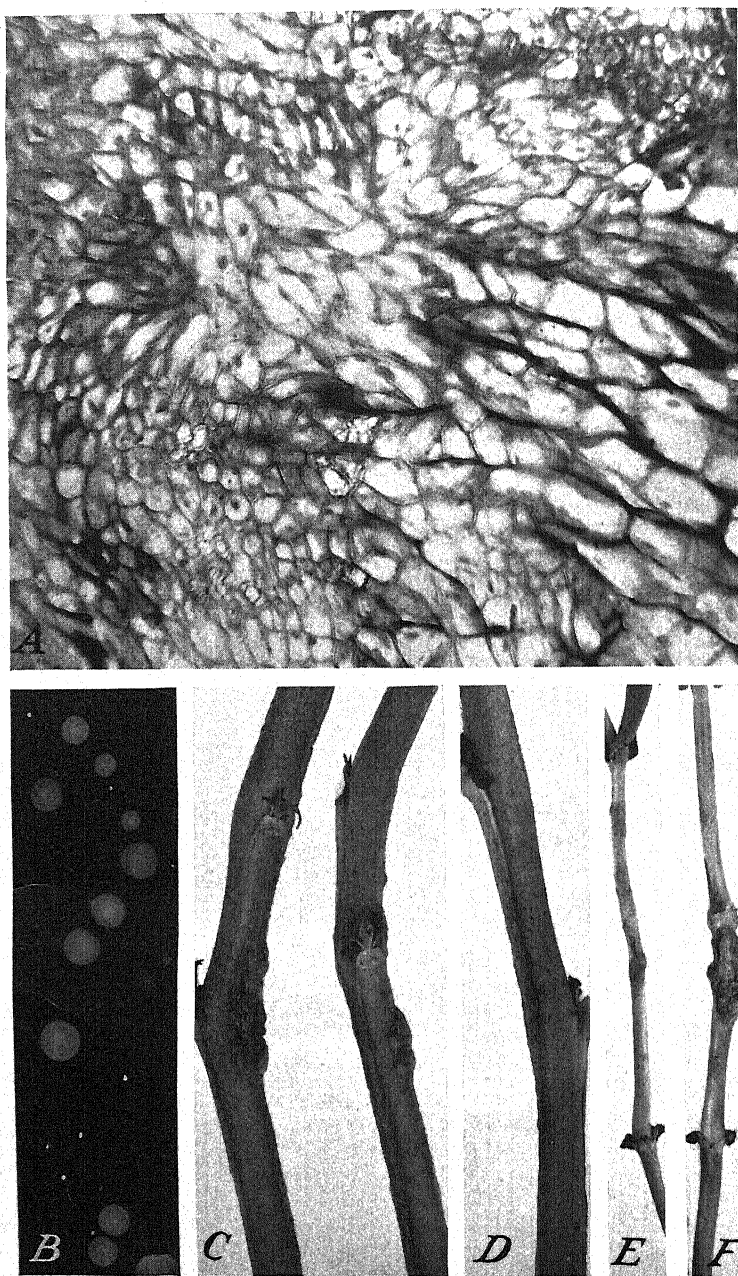
FIGURE 2.—*A*, Cross section of *Gypsophila paniculata* gall showing cell structure.   *B*, Agar-plate colonies of the *Gypsophila* gall organism; natural size.   *C*, Potato stems showing swellings; stems inoculated 3 weeks with *Gypsophila* gall organism that had previously passed through a potato stem and been reisolated; natural size.   *D*, Control punctures on potato stem made at the same time as inoculations in *C*; natural size.   *E*, *Saponaria vaccaria* inoculated with *Gypsophila* gall organism 4 days.   *F*, *S. vaccaria* inoculated with *Gypsophila* gall organism 11 days.   Organism is most active on this host, which is a relative of *Gypsophila*.   *E* and *F* slightly reduced in size.

Roots of seedling *Gypsophila* plants inoculated at the crown likewise gave 100 percent infections. The galls formed about as rapidly as on the older grafted plants (fig. 4, *C, a*). Hot moist conditions favored gall development. Platings were made from the galls produced by inoculation, and the organism was recovered. *Gypsophila* plants inoculated with this reisolated strain developed galls of the same type as rapidly as did plants inoculated with the original strain (fig. 1, *C, a, b, c*).

At the time the *Gypsophila* plants were inoculated, stems of tomato (*Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.), carnations (*Dianthus caryophyllus* L.), garden balsam (*Impatiens balsamina* L.), sugar beets (*Beta vulgaris* L.), Paris daisies (*Chrysanthemum frutescens* L.), nasturtiums (*Tropaeolum* L.), and other plants were inoculated with the same organism. No galls formed on any of them, but well-defined swellings occurred on the potato stems. Isolations were made from the potato swellings, the organism was recovered and outgrowths similar to the natural galls were obtained by inoculating *Gypsophila* plants with this potato isolation (fig. 3, *A*). Potato stems, inoculated with the potato isolation, developed swellings like those caused by the original organism, but neither galls nor cankers (fig. 2, *C, D*). Inoculations made into potato tubers attached to the plant at different stages of growth produced no outgrowths.

The common hosts of *Bacterium tumefaciens*, such as Paris daisy, sugar beet, *Ricinus* L., geranium (*Geranium* L.), and garden balsam, did not prove susceptible to the *Gypsophila* gall organism when they were inoculated with it, nor did *Bact. tumefaciens* produce any trace of outgrowth on roots or stems of *Gypsophila paniculata* (fig. 4, *C, b, c*). The roots and stems of *G. paniculata* were also inoculated with the olive-knot organism (*Bact. savastanoi*) and the ash-canker organism (*Bact. savastanoi* var. *fraxini*) with negative results.

FILTRATES AND PLEOMORPHIC FORMS

The juice of crushed *Gypsophila* galls was passed through Chamberland L 3 filters, and *Gypsophila paniculata* and *Lychnis chalcedonica* L. plants were inoculated with the filtrate. No galls resulted. Filtrates from beef-bouillon cultures were also used for inoculations, with the same result. A portion of the filtrates was held in sterile tubes for several weeks, then cultured repeatedly on hardened agar plates, according to the technic of Hauduroy [3] and of Hadley.[4] With this procedure the filtrates passed through the granular and coccus stages and later reached the normal rod form again, but when the cultures arrived at the rod form the ability to infect the *Gypsophila* plants was lacking. The cultures would not produce galls.

The writer had tried out the method previously with three different strains of *Bacterium tumefaciens*, namely, the hop, daisy, and peach strains. Neither the crushed-gall filtrates nor the beef-bouillon-culture filtrates of the three strains produced galls when inoculated into susceptible plants. Portions of the sterile filtrates were held in tubes for a few weeks to a few months, then cultured for some time on hardened agar plates. From the granules, the coccus form developed, and later from the coccus the normal rods. Inoculations were made into young

[3] HAUDUROY, P. LES ULTRAVIRUS ET LES FORMES FILTRANTES DES MICROBES. 392 pp. Paris. 1929.
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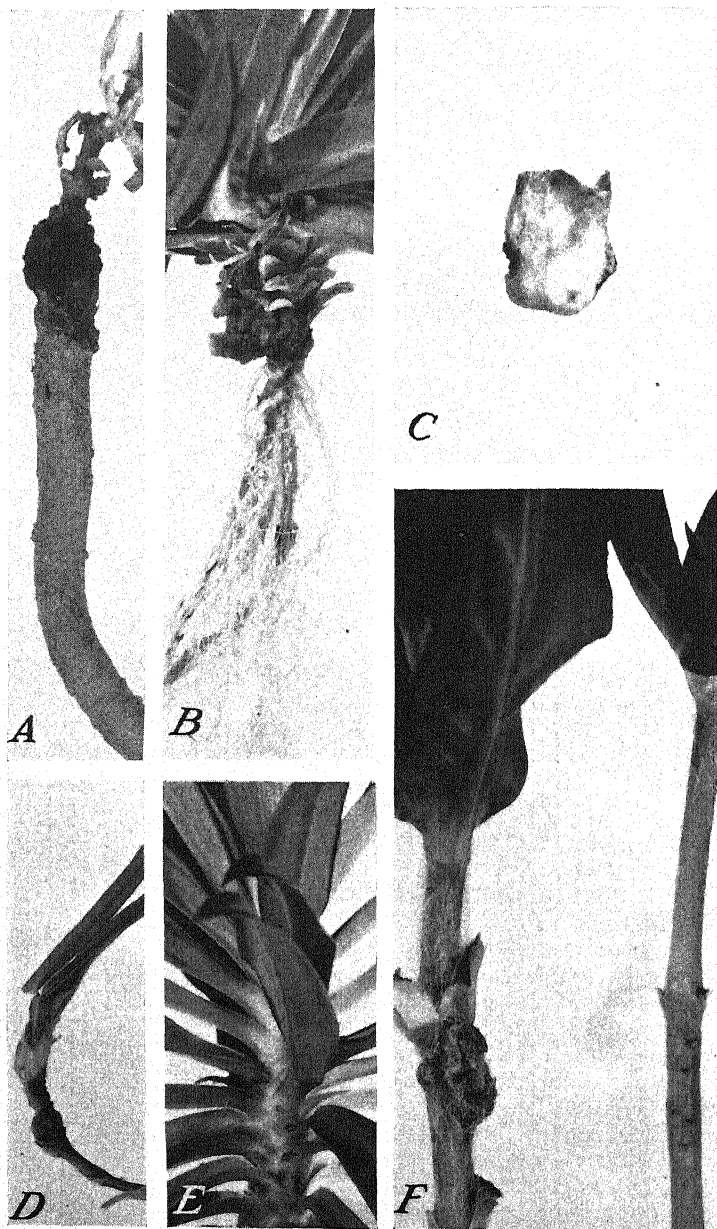
Figure 3.—*A*, Gall on seedling *Gypsophila paniculata*, produced by inoculation with a strain of the organism reisolated from a potato stem on which a swelling was produced but not a gall. The potato reisolation, however, produced galls on *Gypsophila* root. Photographed 5 weeks after inoculation. *B*, Gall on *Silene armeria* produced by inoculation with the *Gypsophila* gall organism; time, 2 months. *C*, *Gypsophila* gall 1 month old cut across to show internal structure. *D*, Gall on *Dianthus plumarius* (garden pink) produced by inoculation with the *Gypsophila* gall organism; time, 2 months. *E*, *Dianthus barbatus* (sweet-william) inoculated with *Gypsophila* gall organism 2 months; no infection. *F*, At left gall on *Lychnis chalcedonica* produced in less than 1 month by inoculation with the *Gypsophila* gall organism; at right, control punctures on *L. chalcedonica*. All natural size.

susceptible plants with both coccus and rod forms, but no trace of crown gall resulted.

With the *Gypsophila* gall filtrates the transition stage from coccus to rod came more quickly than with the filtrates of *Bacterium tumefaciens*, and it was hoped pathogenicity accompanied this less tedious manipulation. It did not prove to be the case, however, as no infection followed inoculations into susceptible plants.

THE CAUSAL ORGANISM

CULTURAL CHARACTERS

BEEF-INFUSION AGAR PLATES.—White colonies are visible in 24 hours after pouring plates from macerated gall tissue incubated at 22° to 25° C. In 48 hours they are deep cream to wax yellow and range from 2 to 4 mm in diameter; they are smooth, circular with entire margin, shining, convex, a little thicker in the center. In 4 days the colonies on thinly sown plates are 4 to 11 mm across, and in some there is a margin. At 6 days the color is mustard yellow; later, primuline yellow.[5]

After the organism has been cultured a few weeks in artificial media, plates poured from a 1-day beef-bouillon culture may show more rough than smooth colonies. These are much the same as the colonies that appear from the isolation material. Occasionally there is a rough colony that has a high convoluted surface. The color of week-old cultures is primuline yellow.

BEEF-INFUSION AGAR SLANTS.—There is a thin spreading growth, usually papillate but sometimes smooth, on beef agar slants in 24 hours. Under the hand lens it has a metallic luster on beef agar. The pH is 6.8 at temperatures of 25° to 30° C. At 4 days growth is abundant, butyrous, translucent; at 7 days the metallic luster has disappeared and there are many crystals. The color of the growth is Naples yellow.

BEEF-INFUSION BOUILLON.—Clouding is prompt, being quite definite in 7 hours at 34° C.; at 30° there is good clouding in 24 hours; in 48 hours a yellow pellicle has formed which falls readily.

THAXTER'S POTATO-DEXTROSE AGAR SLANTS.—The growth is spreading but not so rapid as on beef agar; it is rough, butyrous, cream-colored, and continues so when a week old.

POTATO CYLINDERS.—There is a thin cream-yellow growth in 1 day; it is still scanty at 6 days, with the color buff-yellow, and the potato is slightly discolored. After 30 days the color is Naples yellow, but the medium has not darkened further.

COHN'S SOLUTION.—Growth is rapid and heavy in Cohn's solution, and a complete pellicle forms with larger irregular crystals hanging from it. At first the pellicle is white but changes in 7 days to Naples yellow. The medium becomes cream-colored and has a yellow precipitate.

USCHINSKY'S SOLUTION.—Growth is prompt in this medium, and there is a heavy white pellicle in 2 days; in 12 days the pellicle is cream-colored and the medium Naples yellow.

FERMI'S SOLUTION.—Growth occurs readily but is not so heavy as in Uschinsky's solution. There is a white pellicle in 2 days, which changes to mustard yellow in 12 days.

PHYSIOLOGIC CHARACTERS

LIQUEFACTION OF GELATIN.—No liquefaction begins in beef-gelatin stabs until the cultures are a month old. The liquefaction continues slowly and is not completed until after 4 months. The cultures were kept at 14° to 15° C. and three different lots of beef gelatin, having pH 6.5, 7.0, and 7.3, respectively, were used.

Colonies on beef-gelatin plates are cream-colored until 6 days old, when they become mustard yellow. Both smooth and rough colonies occur on all plates, with the rough type more abundant; they are translucent in reflected light, transparent in transmitted light. In 3 weeks there are little hollows around the colonies, and in 4 weeks liquefaction is definitely visible but continues very slowly. In heavily seeded plates the gelatin is entirely liquefied in 4 months; in sparsely seeded plates the liquefaction extends 1 cm beyond the colony. Feathery branched crystals are formed on the plates and in the stab cultures.

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[5] The color readings in this paper are based on the colors in the following publication: RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D.C. 1912.
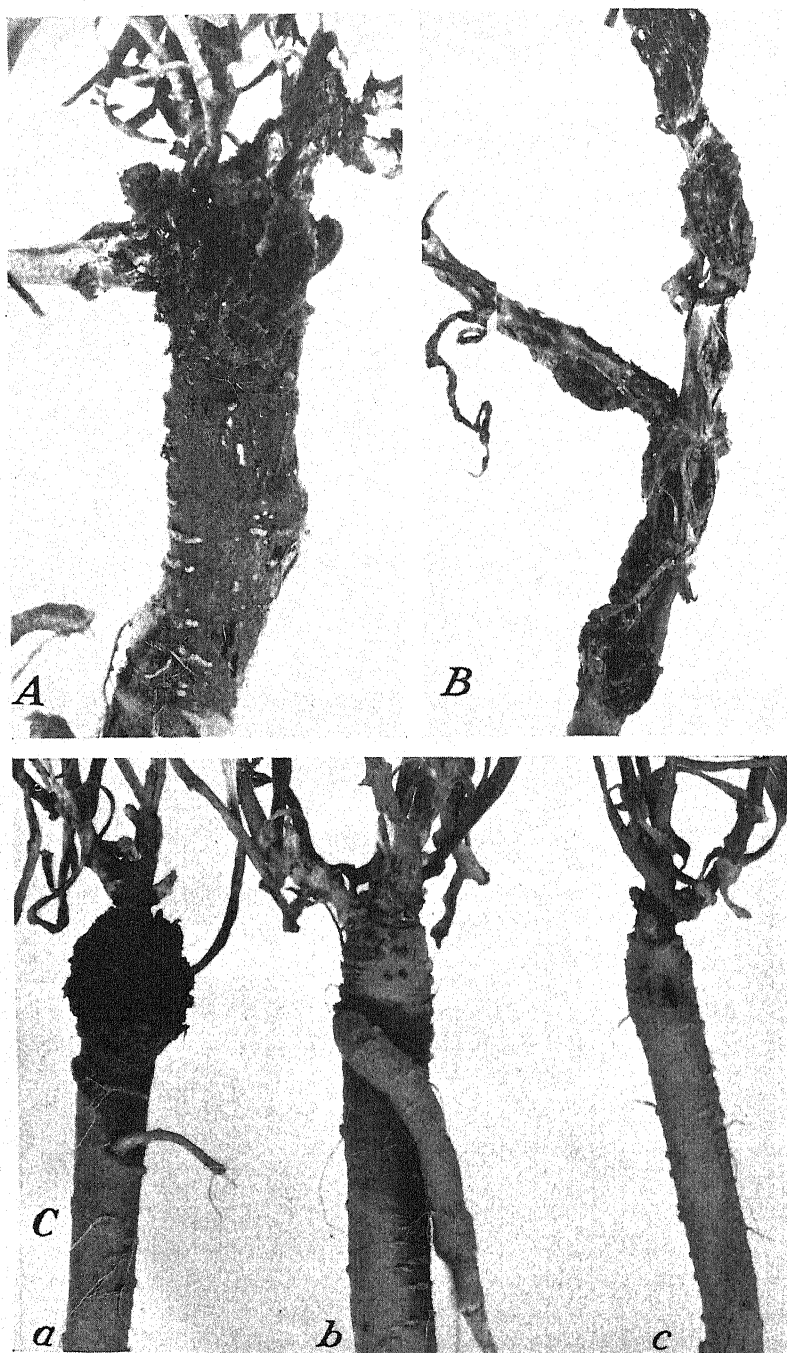
FIGURE 4.—*A*, Gall on *Gypsophila paniculata*, mostly rotted off. Crown was inoculated with *Gypsophila* gall organism July 23, 1932. Photographed August 30, 1932. *B*, Cankers, not galls, produced on *G. paniculata* stems, by inoculation with tne *Gypsophila* gall organism; time, 3 months. *C*, Seedling *G. paniculata* roots inoculated with various organisms 2 months: *a*, with *Gypsophila* gall organism; *b*, with *Bacterium tumefaciens* hop strain, no infection; *c*, with *Bact. tumefaciens* daisy strain, no infection. All natural size.

MILK.—Coagulation of milk occurs at 9 days, with very little whey. A light straw-colored pellicle is visible at 6 days. The curd digests slowly, beginning at 17 days and being completed in 90 days, at which time the color of the milk is tawny.

BLOOD SERUM.—A very good growth takes place on blood serum, but there is no liquefaction. The mustard-yellow color at 2 days becomes the deeper primuline yellow at 12 days. The blood serum grays a little at the base. Cultures 2 months old show no trace of liquefaction.

REDUCTION OF LITMUS.—There is a dull pink color throughout litmus-milk cultures in 24 hours, and the original color, pale aniline lilac, is changed to pale lobelia violet; in 48 hours this color is still duller except at the surface of the liquid. In general the color change in comparison with the uninoculated tubes is slight. In 8 days the litmus has faded to lavender-gray and in 10 days it is reduced, the color now being tilleul-buff; there is a yellow pellicle and yellow precipitate of the growing organism. Coagulation usually takes place on the ninth day, sometimes on the eighth day, after litmus milk has been inoculated. Digestion of the curd is slow, not being completed before 3 months. The medium at this time is dull reddish purple, and the organism is still alive.

REDUCTION OF NITRATES.—Nitrates are reduced to nitrites. Tests were made on nitrate-bouillon cultures 3 and 5 days old, with the sulphanilic acid α-naphthylamine test. There was a good red color in all the tubes, indicating the presence of nitrites. Other cultures when 25 and 30 days old were tested with the same result.

INDOL PRODUCTION.—No indol is produced. Tests were made on the organism growing in tryptophane broth at 3, 5, and 30 days, respectively, with the Ehrlich-Böhme method. *Bacillus coli* (Escherich) Migula, which produces indol, grown as a control and tested at the same time, gave a good pink color, a positive test. The *Gypsophila* gall organism showed no pink color.

FERMENTATION OF CARBOHYDRATES.—The organism is not a gas former. It was tested in fermentation tubes in the presence of the following carbon compounds: Dextrose, saccharose, lactose, glycerin, and mannite. A 1-percent solution of each was made in a 1-percent water solution of Difco peptone. Besides heavy growth in the open arm of each tube there was growth in the closed arm of the tube with each compound except glycerin. No gas was produced. Acid was produced in all the solutions but that of lactose. A second test was made with the same result. The pH readings were taken just before inoculation with the *Gypsophila* gall organism, also 20 and 27 days after, as shown in table 1.

The same carbohydrates added to synthetic agar with brom cresol purple as indicator, made according to the formula given in the Manual of Methods,[6] were also tested for fermentation. Growth occurred promptly, as did the acid reaction with saccharose, dextrose, glycerin, and mannite, the yellow color change in the purple medium beginning in 18 hours and being complete in 48 hours. There was growth in the lactose cultures but no color change.

TABLE 1.—*Acid production by the Gypsophila gall organism after 20 and 27 days of growth in 1-percent sugar solutions added to 1-percent Difco peptone*

[Acidity indicated by pH readings]

Number of days of growth	pH of solution containing peptone and indicated carbohydrate					pH of plain peptone water
	Dextrose	Lactose	Saccharose	Mannite	Glycerin	
0	6.5	6.5	6.7	6.7	6.5	6.6
20	4.2	7.0	5.0	5.0	6.4	7.4
27	4.4	6.8	5.0	4.9	6.5	7.3

DIASTATIC ACTION.—There is no reduction of starch. On starch-agar plates streaked with the organism and tested at 5, 9, 12, and 16 days, respectively, there was no clear zone in the medium.

AMMONIA PRODUCTION.—The organism produces ammonia. Tests were made with old and young beef agar, beef bouillon, and peptone-water cultures, using

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[6] SOCIETY OF AMERICAN BACTERIOLOGISTS, COMMITTEE ON BACTERIOLOGICAL TECHNIQUE. MANUAL OF METHODS FOR PURE CULTURE STUDY OF BACTERIA. 130 pp., illus. Geneva, N.Y. 1930.

filter paper saturated in Nessler's solution and suspended in the tubes. When the cultures were heated in a water-bath, browning of the paper began immediately. In cultures 2 weeks old the brown color was much more pronounced than in the 3- and 5-day cultures.

HYDROGEN SULPHIDE PRODUCTION.—The organism may produce a trace of hydrogen sulphide. Agar, beef-bouillon, and potato-cylinder cultures were tested by suspending lead acetate paper in the culture tubes. When the beef-bouillon cultures were 8 days old there was slight darkening at the tip ends of the paper, indicating hydrogen sulphide production; there was slightly more darkening of the paper when cultures were 2 weeks old. There was no darkening of the paper in the other cultures.

The organism was then grown on lead acetate agar. A heavy yellow growth occurred on the red agar, but there was no dark line or any browning indicating the presence of hydrogen sulphide. A second and third test with the lead acetate agar was made which likewise showed no hydrogen sulphide production.

TOLERATION OF SODIUM CHLORIDE.—The organism grows in pH 6.5 beef-infusion bouillon containing 6, 7, 8, or 9 percent of sodium chloride. There is no growth in beef bouillon containing 10 percent of sodium chloride.

OXYGEN RELATIONS.—The organism is a facultative anaerobe. In tests made with shake cultures of agar and gelatin, tiny clumplike colonies grew throughout the medium. When long sterile cover glasses were dropped on hardened agar plates streaked with the organism, growth was more abundant at the edges of the cover glass, but it extended inside the edges, showing the anaerobic tendencies. In agar and gelatin stab cultures it grew at once at the bottom of the tube, and in synthetic-dextrose-indicator-agar shake cultures, the purple color of the medium was changed to red at the bottom of the tube as quickly as at the surface of the medium.

THERMAL RELATIONS.—The organism grows at temperatures ranging from 5° to 40° C. The optimum temperature is about 34°; it does not grow at 0° nor at 42° and only faintly at 5° and 40°.

The thermal death point is between 52° and 53° C., when fresh beef-bouillon cultures, pH 7.0, are exposed in a water bath for 10 minutes.

GROWTH IN BEEF BOUILLON.—The best growth in peptone-beef-infusion bouillon takes place at pH 6.5 to 6.7, although the organism has a wide range, extending from pH 5.1 to 9. There is no growth at pH 4.9 or 9.1. At pH 5.1 there is only a faint growth; at pH 9 there is a fair amount of clouding with pellicle.

EFFECT OF DESICCATION.—The organism is only slightly resistant to drying. Sterile cover glasses smeared with young beef-bouillon cultures and dried at room temperatures (24° to 27° C.) were dead in 5 days.

EFFECT OF FREEZING.—The organism can withstand freezing temperatures for more than 45 days. Immediately after being transferred, beef-agar and beef-bouillon cultures were placed at temperatures of −21.7° to −23.9° C. Some were removed after 7, 9, 12, and 45 days. All showed typical growth within 1 day after the medium melted.

LONGEVITY.—The organism lives for 7 to 8 months in sterile milk and beef bouillon, pH 6.8, at room temperature of 22° to 30° C., whereas it dies on beef agar slants, in Cohn's, Fermi's, and Uschinsky's solutions, after 4 months at the same temperatures. Sterile-milk and beef-bouillon cultures kept at 14° are alive after 11 months.

VIRULENCE.—The organism remains virulent for more than a year. Fifteen months after isolating, transfers descended from the original isolation, including a smooth and rough colony, were inoculated into *Gypsophila paniculata* plants. In 7 days the galls were forming and continued to grow rapidly.

CHROMOGENESIS.—On beef agar the color of this organism is at first a light cream that changes to a Naples yellow in a few days. It is much the same in other solid media. Later the color may be mustard yellow or primuline yellow.

MORPHOLOGY

Grown on beef-infusion agar, the *Gypsophila* gall organism is a short rod with rounded ends growing singly, in pairs, and occasionally in chains of four to several elements; in rare cases there have been more than 25. Grown on beef agar for 1 day and stained with carbol fuchsin, the size is 0.5μ to 1.2μ long by 0.3μ to 0.8μ wide. Grown on the same medium for 2 days and stained with gentian violet, the

size is 0.4μ to 1.03μ long by 0.2μ to 0.62μ wide. The organism is motile on beef agar and in beef bouillon and its motility was demonstrated by staining with Casares-Gil flagella stain. There are several flagella, all bipolar. Capsules are formed, as was shown by staining young beef agar cultures with Ribbert's capsule stain. The tests for endospores showed none.

STAINING RELATIONS

The organism stains well with gentian violet and carbol fuchsin. It is not acid-fast and is Gram-negative. (Hucker's modification of Gram was used.)

TECHNICAL DESCRIPTION

Bacterium gypsophilae, sp. nov.

A motile rod 0.4μ to 1.2μ long and 0.2μ to 0.8μ wide, with several bipolar flagella; capsules present, no spores; Gram-negative, not acid-fast; colonies on beef-infusion agar are circular, either smooth or rough, yellow, butyrous; clouds beef-infusion bouillon heavily in 18 hours; liquefies gelatin slowly, but not blood serum; is facultative anaerobic; coagulates milk; reduces litmus in 9 to 12 days; grows well in Uschinsky's and Fermi's solutions and unusually well in Cohn's solution; reduces nitrates; produces ammonia and a trace of hydrogen sulphide but no indol; no diastatic action; survives cover-glass drying only 4 days; acid without gas produced with saccharose, dextrose, maltose, mannite, but not lactose, and only a slight amount with glycerin; the optimum temperature for growth is over $30°$ C., the maximum is $40°$, the minimum is $5°$; thermal death point is between $52°$ and $53°$; optimum reaction for growth is from pH 6.5 to 6.7, limits of growth from pH 5.1 to 9.0; in beef bouillon and in sterile milk lives 8 months at $22°$ to $28°$, over 11 months at $14°$; stains readily with carbol fuchsin and gentian violet; pathogenic to *Gypsophila paniculata* and some of its relatives, producing galls on the crown and root, and cankers on the stem.

COMPARISON WITH BACTERIUM BETICOLA

Because of certain points of resemblance between *Bacterium gypsophilae* and *Bact. beticola* and the lesions caused by them, a study of certain cultural, physiologic, and morphologic characters of these organisms was made. A comparison of these characters is shown in table 2.

NATURAL INFECTION AND CONTROL

The *Gypsophila* gall organism is selective in its host plants, as only related plants have been found susceptible to it; however, there are also related plants which are not susceptible. The limitation of this gall-forming ability differs from that of the crown-gall organism, which produces galls on many unrelated plants. The hosts susceptible to the *Gypsophila* gall organism are *Lychnis chalcedonica* (fig. 3, *F*), *Dianthus plumarius* L. (fig. 3, *D*), *Silene armeria* L. (fig. 3, *B*), and *Saponaria vaccaria* L., which is the weed soapwort (fig. 2, *E* and *F*). This last relative seems to be more susceptible to the organism than *Gypsophila paniculata* itself, for infection begins in 3 to 4 days after inoculation and galls are formed very rapidly. It may be that this weed is the natural host of the *Gypsophila* gall organism and it would be advisable not to allow it to grow in the neighborhood of *Gypsophila* plants. Another relative, *Saponaria ocymoides splendens* Hort., is slow to become infected, as the galls did not begin to form on young plants until nearly 3 weeks after inoculation. The relatives, *Cerastium tomentosum* L., *Tunica saxifraga* Scop., *Spergula pilifera* DC., *Dianthus barbatus* L., which is the common sweet-william

(fig. 3, *E*), and the greenhouse carnations are not susceptible. It is an interesting fact that the carnation is not infected by the *Gypsophila* gall organism, for *Bacterium tumefaciens* produces galls thereon quite readily, and occasionally natural *Bact. tumefaciens* galls are found on it. To be quite certain that carnation plants could not be infected by the *Gypsophila* gall organism, inoculations were made at different times of the year and in different growing stages of the plant. Other hosts that did not prove susceptible are sugar beet, tobacco (*Nicotiana tabacum* L.), *Ricinus communis* L., Paris daisy, *Impatiens balsamina*, tomato, geranium, rose (*Rosa* L.), *Bryophyllum pinnatum* Kurz, nasturtium, and two monocotyledons, amaryllis (*Amaryllis* L.) and calla (*Zantedeschia aethiopica* (L.) Spreng.). As stated previously decided swellings but no galls formed on the potato stem.

TABLE 2.—*Comparative cultural, physiologic, and morphologic characters of Bacterium gypsophilae and Bact. beticola*

Character compared	Bacterium gypsophilae	Bacterium beticola
Colonies in beef-agar plates	Circular; none irregular; butyrous; white first day; deep cream to yellow, 4 to 8 mm in diameter, in 4 days.	Circular; some irregular; viscid; buff-colored first day; yellow, 4 to 6 mm in diameter, in 4 days.
Cohn's solution	Rapid, heavy growth	No growth.
Liquefaction of gelatin stabs	Begins after 30 days; complete after 4 months.	Begins after 7 to 8 days; complete in 20 to 30 days.
Hydrogen sulphide production.	A trace to none	Rapid and good production.
Indol production	None	None.
Reduction of nitrates	Nitrates reduced	Nitrates reduced.
Reduction of litmus milk	Complete in 9 to 10 days; milk coagulated in 8 to 9 days.	Complete in 20 to 30 days; milk coagulated in 10 to 20 days.
Gas production	None	None.
Acid produced with dextrose, saccharose, glycerin, and mannite.	Acidity produced	Acidity produced.
Acid produced with lactose	None	None.
Ammonia production	Ammonia produced	Ammonia produced.
Diastatic action	None	Starch reduced.
Relation to oxygen	Facultative anaerobic	Aerobic.
Relation to acid and alkali	pH range 5.1 to 9.0	pH range 4.8 to 9.1.
Temperature relations	Grows from 5° to 40° C; optimum, about 34°; thermal death point, 52° to 53°.	Grows from 1.5° to 39° C.; optimum, about 29°; thermal death point, 51° to 52°.
Survives cover-glass drying	4 days	7 days.
Color	Ranges from white to yellow	Ranges from buff to yellow.
Size	0.4μ to 1.2μ long; 0.2μ to 0.8μ wide	0.6μ to 2μ long; 0.4μ to 0.8μ wide.
Gram negative or positive	Negative	Variable.
Pathogenicity	Produces galls on *Gypsophila paniculata* but not on sugar beets.	Produces galls on sugar beets but not on *Gypsophila paniculata*.

The *Gypsophila* gall organism produces cankers on stems of *Gypsophila paniculata*, fair-sized cankers forming in less than 1 month after inoculation, large ones in 3 months (fig. 4, *B*). On *Lychnis chalcedonica* stems the infection is of the typical gall type. *Lychnis* crowns inoculated in November in the greenhouse developed galls, which rotted away during the winter. In the spring, when growth started, new galls formed which became larger than the original ones. This occurred with inoculated *G. paniculata* plants also, although the galls were not so soft and did not disintegrate so easily. The organism, like other organisms that produce galls, is a wound parasite, this one getting into the plant from the soil through imperfect grafting or through cultivation wounds. The disease occurs on lands that have been manured and on those that have not.

The rapid development of galls at the crown produces the death of some of the stems, and if the girdling is complete the death of

the entire plant follows. A small gall which has not caused any apparent trouble to the plant may be a decided menace to others later. The method used for propagating *Gypsophila* plants is to graft a desirable variety on seedling roots. If this variety is galled the organism is carried over to the young seedling roots and galls develop. Because of the sensitiveness of the gall organism to weak solutions of mercuric chloride, control measures can be carried on at the time of grafting to reduce the amount of disease occurring in the field. The roots should be dipped in a 1:1,000 mercuric chloride solution for 1½ to 2 minutes to kill the *Gypsophila* gall bacteria that may be on the surface; then with a disinfected knife a well-matched graft should be made and bound with nursery tape.

The sensitiveness of the organism to weak solutions of mercuric chloride was tested out by the poured-plate method. A fresh beef-bouillon transfer was exposed to 1 cc of a 1:1,000 solution of mercuric chloride for various lengths of time and then plates were poured, carrying over a loop of the exposed culture to each agar plate. No *Gypsophila* gall colonies appeared on the plates exposed over 1½ minutes to the mercuric chloride solution. As *Gypsophila paniculata* plants are not very sensitive to a 1:1,000 solution of mercuric chloride they can be treated with it to kill the gall organisms that may be present on the surface. Twenty-five seedling plants 3 to 6 inches tall were soaked in the solution for 1½ minutes, 25 for 3 minutes, 25 for 5 minutes, and 25 for 10 minutes. There was no appreciable injury to the plants from this treatment, even in the 10-minute lot.

In planting out seedlings and grafted plants, in weeding them and in loosening the soil about them during the summer, care should be taken to avoid wounding, for the organism may be present in the soil and may enter the plant through some tiny wound.

SUMMARY

An outbreak of an infectious gall disease occurred in 1932 in an ornamental-plant nursery in the eastern part of the United States. The galls were on the crown and roots of *Gypsophila paniculata*. They were soft and nodular; some were flat and spreading, others globular.

The *Gypsophila* galls when developing do not have the fissures or pockets that occur in developing galls of pocket disease of sugar beets and in those of olive knot. Growth of the galls is favored by hot moist weather, which also favors the disintegration of the old galls, releasing more organisms into the soil. Imperfect grafting and cultivation wounds allow entrance of the pathogene into susceptible tissue. Galls weaken the plants, producing defoliation and death of stems and, where girdling is severe, death of the plants.

An organism, which could be seen readily in sound gall tissue under the microscope, was isolated from the outgrowths and produced galls when inoculated into healthy plants of *Gypsophila paniculata*.

Galls were also produced by inoculation on several species related to *Gypsophila*, but the organism did not produce galls on the sugar beet, which is the host of the pocket disease, nor on such common hosts of the crown-gall organism as the Paris daisy, *Ricinus*, or geranium.

For the pathogene, which is a yellow, polar-flagellate organism apparently unlike any other known gall-producing organism, the name *Bacterium gypsophilae* is proposed. A description of its cultural, physiologic, and morphologic characters is given.

A comparison has been made between the new gall organism and *Bacterium beticola*, the organism causing the pocket disease of sugar beets.

A study has been made of conditions governing the natural occurrence of infection, and methods of controlling the disease are suggested.

EXPERIMENTS ON IAROVIZING CORN [1]

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INTRODUCTION

The methods of iarovization advocated by the Russian workers fall into two distinct groups: The low- and the high-temperature treatments. The low-temperature treatments have been known for a long time and are well established experimentally, as is evidenced by the work of Klippart (*3, p. 757*),[3] Lysenko (*4*), McKinney and Sando (*6*), and others. The high-temperature treatments are less well established. They have been advocated as a very effective agent in the hastening of sexual maturity in the short-day plants. If the results reported by the Russians are universally confirmed, the process of iarovization might be expected to play a very important role in certain temperate regions. Iarovization of corn in the United States, however, even if effective, does not appear to offer any great commercial possibilities. On the other hand, it might have considerable value in certain physiologic and genetic experiments.

THE PROCESS OF IAROVIZATION

Originally the term "iarovization" (vernalization) was applied only to the low-temperature treatment of winter wheat to induce jointing and hasten sexual maturity. At the present time the term is generally applied to any treatment having as its object the hastening of sexual maturity.

The requirements for different crops differ in the duration of the treatment, the temperature, and the moisture content of the seeds. A summary table is presented by McKinney and Sando (*7*) listing the requirements for a few crops. All of the high-temperature treatments, according to the Russian workers, must take place in darkness.

MATERIALS AND METHODS

The experiments reported in this paper were conducted at the Arlington Experiment Farm, Rosslyn, Va., in 1933. Eight hybrids, 9 inbreds, and 1 variety of corn and 1 strain of teosinte were included. The variety of corn used was Gaspé, one of the earliest known. The hybrids and 7 of the inbreds are adapted to Corn Belt conditions. Of the remaining inbreds one is a derivative of the Garrick variety which is adapted to the South, and the other is a type called "Cuzcoid" because of its resemblance to the varieties from Cuzco, Peru. This last is a simple Mendelian recessive and in the segregating prog-

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enies so far tested has required about twice as long to reach the reproductive stage as its normal sibs. Sexual maturity in this strain has not been hastened by exposure to a 10-hour day.

A further indication of the range of season represented by the inbreds in this test may be obtained from the following comparison. The earliest strain, 616, shed pollen 60 days after planting. Cuzcoid, the latest strain, was killed by frost October 26, 160 days after planting, when less than one-tenth of the plants had tasseled. At this time it had an average of 38 nodes per plant.

All of the seed used was soaked in a 0.5-percent solution of Uspulun for 2 hours and then rinsed. They were then soaked for 9 hours in a weak salt solution of about the same molecular concentration as tap water and were then dried to approximately the moisture content (30 percent) recommended. This method was found to result in much more uniform germination than adding stated quantities of water as recommended by Lysenko (*4*). One complete set of the samples was then placed in light-proof bottles in a constant-temperature chamber and held at 75° F. for 14 days. Duplicates of some of the samples were subjected to the same temperature conditions, but exposed to the normal day or to continuous light (normal day plus artificial illumination). One lot was held at a temperature of 38° F. for the same period. At the end of the 14-day period all of the samples were planted in the field. In those samples in which germination had progressed the farthest the radicles were approximately 5 to 8 mm and the plumules 5 mm long. Molds, particularly *Penicillium*, occurred in some seed lots, but no visibly infected seeds were planted.

The iarovized material was hill-checked in some cases in comparison with sprouted seed, and in other cases with dry seed of the same sort. In some instances, the difference due to the slight hastening of emergence because of the sprouted condition of the iarovized seed persisted and was reflected in a slightly earlier tasseling and silking (table 2, items 3 and 6). This should not be a serious source of error in experiments of this kind, as a slight advantage from this cause would be insignificant as compared with the marked acceleration which must result from iarovization if the method is to be commercially practical.

Dates of germination, pollen shedding, and silking were recorded for the plants in all perfect hills. In addition, percentage of germination, number of nodes, plant heights, and plant yields were obtained in most cases. All of the data reported are based on comparisons between plants of the same strain growing in the same hill.

EXPERIMENTAL RESULTS

The effect of the treatments on field germination and on emergence of the inbreds is shown in table 1. In every case iarovization resulted in a marked reduction in germination and in 13 of the 14 cases delayed emergence. The germination of the iarovized seed was so poor and seedling mortality following germination so great that no adequate data on maturity are available. For the three strains A, 324, and 540, which had the highest germination percentage and for which meager data are available, there was no indication of a hastened maturity following the treatments.

TABLE 1.—*Effects of iarovization on field germination and emergence in 14 inbred strains of corn*

Inbred strain no.	Condition of check	Germination		Mean difference between iarovized and check seed lots in time to emergence[a]
		Iarovized	Check	
		Percent	*Percent*	*Days*
616	Sprouted	35. 0	97. 5	1. 4
A	do	70. 0	97. 5	1. 3
324	do	75. 0	100. 0	. 9
420	do	55. 0	95. 0	2. 0
420 [b]	do	32. 5	97. 5	1. 7
420 [c]	do	7. 5	97. 5	2. 5
461	do	22. 5	97. 5	1. 8
461 [b]	do	25. 0	95. 0	1. 7
461 [c]	do	40. 0	100. 0	2. 0
461	Dry	20. 0	97. 5	1. 7
540	Sprouted	80. 0	97. 5	. 3
119–11–b	do	22. 5	92. 5	2. 0
207–37	do	35. 0	72. 5	−. 4
Cuzcoid	do	54. 0	83. 0	+. 5

[a] Positive differences indicate the iarovized lot emerged later than the check. In the column headed emergence a positive difference indicates that the iarovized lot required more days to emerge than the control.
[b] Iarovized in light (day only).
[c] Iarovized in light (continuous).

With the more vigorous material, which includes the hybrids and the variety Gaspé, there was a slight but consistent reduction in germination as a result of iarovization, as shown in table 2. The time required for germination shows no consistent differences resulting from the treatments. Only three of the mean differences are significant. In two of these, iarovization appears to have had an accelerating effect, but in both the check seed was dry and ungerminated. In one instance there is a significant retardation. In this paper, differences have been considered statistically significant when P is 0.02 or less. Such differences are italicized in table 2.

In 8 of the 15 comparisons, plants from iarovized seed shed pollen significantly before the checks, but in only 5 cases was there a significant difference in silking. This is in accord with general observations that pollen shedding is influenced to a greater extent by environment than is silking. There is a fairly high positive correlation between the days required for emergence and for pollen shedding. Substantially the same degree of correlation exists in both the iarovized and control lots. It should perhaps be emphasized that while in several cases hastening of sexual maturity is statistically significant, in no case is the acceleration of any significance agronomically.

The numbers of nodes visible at maturity were fewer in the plants from iarovized seed in all of the 11 comparisons involving iarovized *v.* noniarovized seed and of which counts were made. Only 7 of the 11 differences are statistically significant, but 11 deviations of like sign would be expected only once in 2,048 trials as a result of sampling error alone. Counting nodes visible at maturity is not satisfactory where the absolute number is wanted. In the present case, however, the interest lies in the relative difference between paired plants of successive hills, and the visible number of nodes is just as satisfactory a basis for comparison as the absolute number.

TABLE 2.—*The effects of iarovization on various agronomic characters in 8 hybrids and 1 variety of corn*

[Differences for which *P* is less than 0.02 are italicized]

Hybrid or variety	Condition of check	Field germination		Mean differences between iarovized and control plantings in—a						
				Time to—			Nodes	Plant height	Ears	Weight of shelled grain per plant
		Iarovized	Check	Emergence	Pollen shedding	Silking				
		Pct.	*Pct.*	*Days*	*Days*	*Days*	*No.*	*Inches*	*No.*	*Grams*
A×325	Sprouted	87.5	95.0	0.8	−0.7	0.3	−1.9	−9	−0.1	−14.8
A×164	Dry	95.0	100.0	−.4	−3.0	−1.9	−1.8	−7	.0	−48.8
A×164 b	do	100.0	100.0	−1.0	−1.7	−1.6	−.3	−9	−.1	−18.7
A×164	Sprouted	92.5	100.0	.0	−2.0	−1.2	−1.6	−5	−.1	−44.5
420×164	Dry	72.5	95.0	.0	−.6	−.4	−1.1	−4	−.6	−40.0
420×164 b	do	95.0	97.5	−1.0	−2.1	−1.3	−.3	−3	−.1	−8.9
420×164	Sprouted	85.0	87.5	.6	.6	.7	−1.0	−3	−.3	−28.1
420×4–8	do	97.5	100.0	.0	−1.0	−.5	−1.2	−3	.0	−38.0
325×420	do	100.0	100.0	−.1	−1.7	−.9	−.8	−3	.0	−30.1
325×420 c	do	95.0	100.0	.0	−2.7	−1.9	−1.0	−3	.0	−1.9
540×164	do	95.0	100.0	.6	.1	.2	−.8	−4	−.1	−23.9
L×317	do	97.5	100.0	−.5	−1.6	−1.0	−.7	−3	.0	−10.3
317×461	do	100.0	97.5	.4	−.3	−.2	−.7	1	−.3	−23.7
Gaspé	do	95.0	100.0	*1.5*	.7	.6				
Do. d	do	95.0	97.5	.6	−.3	−.5				

a Positive difference indicates iarovized lot exceeds check.
b Sprouted, not iarovized.
c Iarovized, continuous light.
d Iarovized at 38° F.

There appears to be no consistent relationship between the reduction in number of nodes and plant height as a result of the treatment. In some strains the iarovized plants exhibit a significant reduction in number of nodes and yet are not significantly shorter than their controls.

Where any differences exist in number of ears, the iarovized material always has the lower number. None of the differences, however, is significant.

All of the iarovized material exhibits a reduced yield of shelled grain per plant, only five of the comparisons, however, being significant. The reductions in yield indicated in table 2 as being significant represent a reduction of approximately 15 to 30 percent. It is worthy of mention that the comparison exhibiting the least reduction in yield was significantly earlier in pollen shedding and silking than its controls and was iarovized under continuous light.

In the variety Gaspé iarovization at 75° F. or at 38° F. for a 14-day period was ineffective in hastening sexual maturity.

DARKNESS REQUIREMENT

According to the theory advanced by Lysenko (*4*), short-day plants require light for processes of growth and the absence of light (darkness) for the initiation of reproduction. The necessary darkness may be supplied continuously during the early stages of the plant's development or as periods alternating with light, as in day and night, during a greater portion of the plant's development. Iarovization carried on in the dark is presumed to be effective, in the case of short-day plants, because it satisfies the plant's requirement for

darkness. With this requirement satisfied, the plants are able to benefit from the long days, and hastened sexual maturity results.

The theory of a "darkness requirement" for the initiation of reproduction in corn, at least for some varieties of the temperate regions, is not in agreement with several facts. Corn has been grown in the greenhouse at the Arlington Farm during two winters under continuous illumination (normal day plus artificial illumination) without any material delay in the onset of flowering or maturity. In the winter of 1932–33 four inbred strains representing a considerable portion of the seasonal range of Corn Belt varieties were grown under continuous illumination from the dry seed to maturity. The plants were perfectly normal in their vegetative development and seasonal maturity.

As a further test for the necessity of a dark period, the same strains of corn were grown during the summer, one set of plants being exposed to the normal day, and a second set to continuous illumination (normal day plus artificial illumination). The results are presented in table 3.

TABLE 3.—*Effect of day length on sexual maturity in 4 inbred strains of corn*

Inbred strain no.	Period from planting till pollen shedding when grown with a day length of—		
	12 hours	16 hours	24 hours
	Days	*Days*	*Days*
616		52	60
A		55	61
228-4-8	61	62	69
461		60	66

Continuous illumination resulted in approximately a week's delay in pollen shedding. For the one strain grown also with a 12-hour day, there was no significant difference in earliness between the 12-hour and 16-hour photoperiods.

The classification of corn as a short-day plant does not appear to be based on adequate experimental evidence. It is true that the work of Garner and Allard (*2*), Emerson (*1*), and McClelland (*5*) has shown that some varieties respond to a short day (10 to 12 hours of light). However, all of the varieties used by these investigators were tropical or semitropical sorts naturally adapted to short-day conditions. Varieties which are adapted to Corn Belt or more northern conditions and which normally bloom during a long day (15 to 18 hours of daylight) have not been adequately studied. In three strains grown in the greenhouse, augmenting the winter day (11 to 13 hours) by artificial illumination for 4.5 or 8.5 hours has not resulted in a significantly delayed sexual maturity.

Teosinte has been shown by Emerson (*1*) to respond to a short day. It was thought that a comparison of the effectiveness of darkness supplied to this plant during the iarovization process and as a daily photoperiod would be instructive. Three lots of teosinte were planted May 19, one of them having been iarovized. The iarovized lot and one of the others were exposed to the natural day. There

was no significant difference in sexual maturity of these two lots, both showing tassels September 16 and shedding pollen October 3. The second noniarovized lot was exposed to a 10-hour day. It showed tassels June 6 and shed pollen June 10, having been exposed to fewer hours of darkness (328) during this period than had the iarovized lot (336 hours) during the period of iarovization. It seems clear from this that length of day is much more important in determining the time of sexual maturity than is any darkness requirement that teosinte may possess (fig. 1).

McKinney and Sando (8) have shown that after the iarovization treatment the attainment of sexual maturity in winter wheat is greatly influenced by day length. To determine whether a similar condition exists in short-day crops, a second set of material, common millet and the corn hybrid A×164, were iarovized at 80° F. at the moisture contents and for the periods recommended, one lot of seed of each crop in continuous darkness and a duplicate lot in continuous light (normal day plus artificial illumination). Following iarovization, the various lots, including dry and germinated checks, were grown in pots under a 16-hour and a 24-hour day.

FIGURE 1.—Response of teosinte to darkness. The plant on the right was exposed to darkness for a 14-hour period daily. The one on the left was exposed to darkness continuously for 14 days followed by exposure to darkness for an 8- to 9-hour period daily. Photographed June 19, 31 days after planting.

The treatment given the millet was ineffective in hastening sexual maturity under either light treatment. The corn plants receiving the 16-hour day responded approximately as they had done in the field. For the plants grown under continuous illumination, iarovization with continuous light was superior to iarovization with continuous darkness in promoting early flowering, and both lots from iarovized seed were earlier than those from either the germinated or dry checks. The results are presented in table 4. There is nothing in the results obtained in these experiments with corn and teosinte to lend support to the darkness-requirement theory of Lysenko.

TABLE 4.—*Influence of day length on the attainment of sexual maturity in corn*

Photoperiod (hours)	Period required to attain sexual maturity under indicated treatments			
	Iarovized		Control	
	Continuous dark	Continuous light	Germinated	Dry
	Days	*Days*	*Days*	*Days*
16	64	61	65	67
24	76	71	80	81

DISCUSSION

The iarovization of certain corn hybrids resulted in a statistically significant hastening of sexual maturity. The difference, however, was so slight as to be of no importance agronomically. The general reduction in germination and vigor associated with the iarovization treatment appears to be a serious limitation of the method. In this respect these results depart rather drastically from those reported by the Russian investigators. The reason for the failure of agreement with their results is not entirely clear.

The Russians emphasize the fact that varieties differ markedly in their iarovization requirements. It is possible that all of the strains used in these tests require special conditions during iarovization, though this does not seem likely.

The experiments of McKinney and Sando (*8*) and the results reported here (table 4) are in agreement in indicating that the day length following iarovization has an important effect on the reaction. The plants in the present studies (tables 1 and 2) were grown under a daily photoperiod of approximately 15 hours, and those in the Russian work presumably under a longer daily photoperiod. It seems probable that at least part of the difference between the results in the two places may be ascribed to the day length under which the plants were grown. The claims for the necessity of darkness during the iarovization process, however, are not substantiated by either the field or greenhouse tests.

SUMMARY

Iarovization of corn, as practiced, consistently reduced the percentage of germination. It also resulted in a general reduction in the number of visible nodes, in plant height, number of ears, and weight of shelled grain per plant.

Pollen shedding and silking were significantly accelerated by iarovization in some strains but not to an extent to be of any agronomic importance.

There was no evidence of a darkness requirement for corn. Several strains of corn were grown to maturity under continuous light. Iarovization in continuous light was just as effective as that in continuous darkness.

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THE MICROBIAL DECOMPOSITION OF SUCCESSIVE CUTTINGS OF ALFALFA HAY UNDER AEROBIC CONDITIONS [1]

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INTRODUCTION

The present investigation was undertaken to determine whether there is any difference in the decomposition of successive cuttings of alfalfa hay aerobically fermented by soil micro-organisms.

A review of the literature gives little information regarding the influence of chemical composition on the microbial decomposition of successive cuttings of alfalfa hay.

Falck and Haag (*1*)[2] concluded from their studies that in the microbiological decomposition of plant materials two distinct processes take place, namely, destruction and corrosion. The effect of destruction is to decompose the cellulose and pentosans, the lignins being very little affected. Corrosion, on the other hand, causes slow decomposition of both lignin and cellulose.

Starkey (*3, pp. 293–294*) stated:

When the decomposition of organic matter is measured by the amount of CO_2 produced, it should be kept in mind that the CO_2 is not the only product formed from the carbon of the organic matter * * * since various intermediate products may be formed * * *. In general, however, the incomplete decomposition products of some organisms are further attacked by others and sooner or later appear as CO_2. * * * The CO_2 produced from soils should, therefore, give a reliable although not an absolute index of the decomposition of organic matter.

Waksman and Tenney (*6*) found that when a comparison is made of the rapidity of decomposition of a plant which has been harvested at different stages of growth, different results are obtained. The more mature the plant is the less readily does it decompose. Their analysis showed that a third of all the constituents of the young rye plant, on a dry basis, consisted of water-soluble substances, including considerable quantities of sugars and amino acids. The young plant contained, on a dry basis, 2.5 percent of nitrogen, 7.7 of ash, 16.6 of pentosan, 18.06 of cellulose, and 9.9 percent of lignin. With the advance in the age of the plant, there was a rapid decrease in the nitrogen and in the fat and ash content and a gradual increase in the cellulose, pentosan, and lignin content. There was considerable decrease in the amount of water-soluble constituents. The mature plant (exclusive of the grain and roots) contained 0.24 percent of nitrogen, 22.9 percent of pentosan, 36.3 percent of cellulose, 19.8 percent of lignin, and 9.9 percent of water-soluble substances.

EXPERIMENTAL METHODS

In order to determine differences in the microbial decomposition of successive cuttings of alfalfa hay, two experiments, each in duplicate, were conducted. Three cuttings of hay were collected from each of

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the 1931 and 1932 crops under similar conditions but from different fields. The hay used in the first experiment (1931 crop) was collected from a field on the United States Department of Agriculture Experiment Farm at Beltsville, Md., on which heavy manuring and intensive cultivation methods were used. The hay used in the second experiment (1932 crop) was collected from a privately owned field on which only ordinary fertilizing and cultivation methods were used. As Van der Spuy and Stead (4) have shown by analyses of plants at various stages of growth that the nutritive value of lucerne hay is highest when the hay is cut at the 10-percent stage of flowering, and also that the highest yield of hay is obtained by cutting at this stage of growth, the alfalfa hay for each experiment was collected when most of the plants were in partial bloom. Each cutting was obtained from the
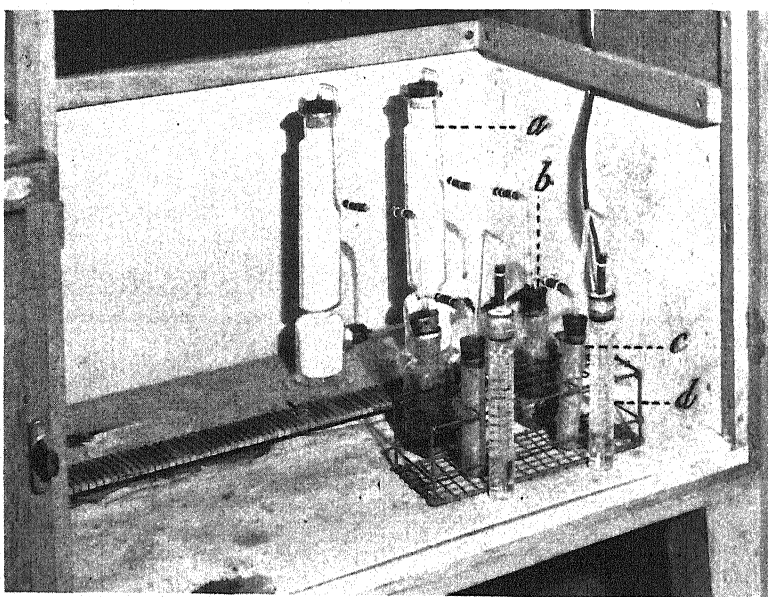
FIGURE 1.—Aerating apparatus for the determination of carbon dioxide in fermenting plant materials, a, Soda lime tower; b, humidifier; c, fermentation tube; d, spiral absorber.

same area in each field in order that environmental growth factors would be the same. Immediately after cutting, the material was sundried for several days and then passed through a grinder.

In the first experiment, two 10-gram samples (calculated on a dry basis) of each of the three cuttings were inoculated with sufficient soil infusion to give a total moisture content of 43 percent. In the second experiment, two 6.5-gram samples of each cutting were similarly prepared. The soil infusion used in each experiment was prepared by mixing 100 grams of soil, obtained from the same area in the field from which each cutting of hay was obtained, with 150 cubic centimeters of water and filtering through sterile cotton. The filtrate was thoroughly shaken and used as the inoculum for each of the three cuttings of hay.

The inoculated hay samples were placed in large test tubes fitted with aeration tubes extending to the bottoms, connected to an aerat-

ing apparatus, and incubated at 30° C. for 30 days. Each aerating apparatus (fig. 1) consisted of a soda lime tower, *a*, containing wet pieces of sponge for moistening the air before it was passed through the hay sample; a fermentation tube, *c*, containing the hay sample; and a spiral absorbing tube, *d*, containing potassium hydroxide (*2*) to absorb the carbon dioxide produced from the fermenting hay.

Air was passed through the apparatus from a pressure line connected to a large "bleeder" bottle and then to the soda lime tower. The "bleeder" bottle was necessary to reduce the pressure and also to adjust the air flow at any desired rate. It consisted of a 15-liter bottle equipped with inlet and outlet tubes and a stopcock through which the excess pressure was released. The air flow was regulated at such a rate that the gas bubbles passing through the spiral tube containing the potassium hydroxide came in contactwith the absorbing liquid long enough to allow for complete absorption. This rate of flow was found to be approximately 2 liters of air per hour. Determinations were made to check the efficiency of the soda lime towers and spiral absorbers. Daily titrations of the potassium hydroxide were made, and the quantities of carbon dioxide evolved from the fermenting hay were determined and recorded as milligrams of carbon.

Oven-dried samples from the three cuttings of the 1931 crop were analyzed for the important chemical fractions by the modified method of Waksman and Tenney (*7*).

FIGURE 2.—Total carbon dioxide evolved during the microbial decomposition of three cuttings of alfalfa hay from the 1931 crop.

Owing to limited facilities, the hemicelluloses, cellulose, and lignin were not directly determined but were recorded as the fraction soluble in 2-percent HCl, the fraction soluble in 80-percent H_2SO_4, and the residue, respectively. At the end of the fermentation period, the material remaining was dried to constant weight at 105° C. From the results obtained by the difference in weight before and after fermentation the percentage loss due to microbial action was determined. The oven-dried material was then analyzed as previously mentioned. Each determination was calculated on the basis of the original weight of material used, thereby showing the actual percentage loss of each fracton caused by the microbial decomposition. The chemical analyses on both the unfermented and fermented hays were repeated several times in duplicate.
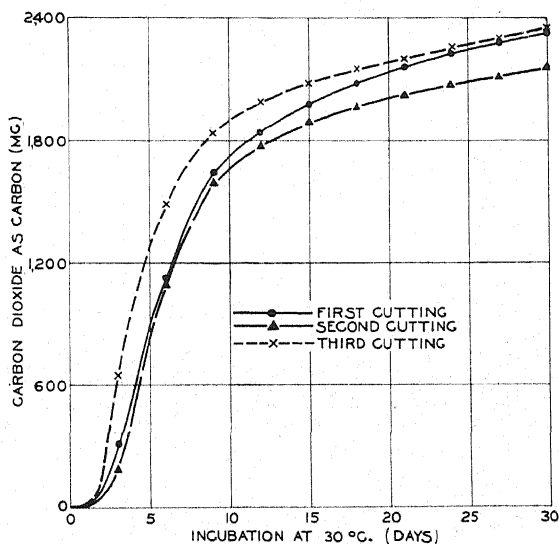
RESULTS

Results on the total quantities of carbon dioxide evolved during the microbial decomposition of three cuttings of alfalfa hay from the 1931 and 1932 crops are shown in figures 2 and 3. In both experiments (figs. 2 and 3), the third cutting showed the greatest decomposition, the first cutting was next in order, and the second cutting showed the least decomposition. Results on the daily titrations of carbon dioxide evolved during the microbial decomposition of three cuttings of alfalfa hay from the 1931 crop are shown in figure 4.

It will be noted that the greatest variation in the rate of decomposition of the three cuttings of hay occurred during the first 10 to 12 days of the fermentation, after which it gradually decreased to the end of the test period. Similar results were

FIGURE 3.—Total carbon dioxide evolved during the microbial decomposition of three cuttings of alfalfa hay from the 1932 crop.

noted in the second experiment (1932 crop). At the end of the first experiment, the percentage loss of dry matter due to microbial decomposition was 41.3 for the third cutting, 39.2 for the first, and 35.8 for the second. In the second experiment, the percentage loss was 43.6 for the third cutting, 41.4 for the first, and 37.3 for the second.

Chemical analyses were made on oven-dried samples from the three cuttings of hay used in the first experiment before and after microbial decomposition. The results shown in table 1 were included to give the approximate chemical
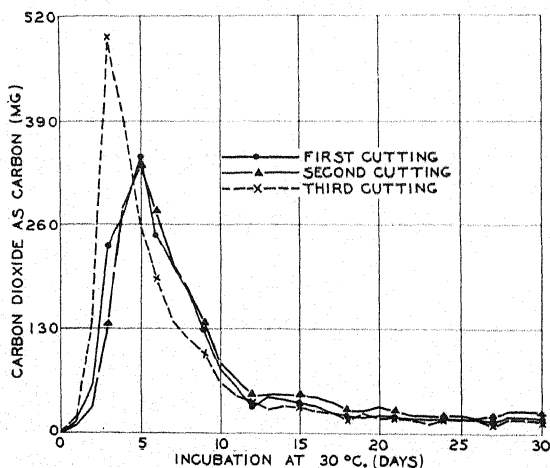
FIGURE 4.—Daily quantities of carbon dioxide evolved during the microbial decomposition of three cuttings of alfalfa hay from the 1931 crop.

composition of each cutting of hay before fermentation and similar data after fermentation, showing the percent loss in each of the fractions. It is noted that in the larger fractions (cold water, 2-percent HCl, and 80-percent H_2SO_4), although the percentages in the original hays were approximately the same in each cutting, the percentage losses after fermentation varied. According to Waksman and Tenney (5), the various fractions were composed of the following constituents: (1) The ether fraction contained fats and waxes; (2) the cold-water fraction contained simple carbohydrates, various amino acids, peptides, and soluble minerals; (3) the hot-water fraction contained starches, pectins, certain hexosans, and various nitrogenous compounds; (4) the 2-percent HCl fraction contained hemicelluloses and protein; (5) the 80-percent H_2SO_4 fraction contained cellulose and protein; and (6) the residue contained lignin, protein, and ash.

TABLE 1.—*Percentage composition (by fractions containing various constituents) of successive cuttings of alfalfa hay from the 1931 crop before and after microbial decomposition, and percentage loss of each fraction*

[On moisture-free basis]

Chemical fraction	First cutting			Second cutting			Third cutting		
	Original material	After 30 days' decomposition		Original material	After 30 days' decomposition		Original material	After 30 days' decomposition	
		Calculated on original weight	Loss		Calculated on original weight	Loss		Calculated on original weight	Loss
Ether	2.0	1.7	15.0	3.0	2.7	10.0	2.2	1.7	22.7
Cold-water	24.6	10.3	58.1	24.2	13.7	43.3	26.7	13.2	50.5
Hot-water	5.1	3.2	37.2	4.2	4.0	4.7	4.6	2.8	39.1
2 percent HCl	28.6	17.3	39.5	29.2	15.8	45.8	30.3	17.4	42.5
80 percent H_2SO_4	23.3	15.4	33.9	22.2	14.0	36.9	19.4	9.8	49.4
Residue	14.0	11.0	21.4	13.6	11.9	12.5	13.3	12.0	9.7
Protein, insoluble in cold H_2O [a]	12.3	7.3	40.6	13.2	7.2	45.4	15.5	8.6	44.5
Total nitrogen [a]	2.5	1.5	40.0	3.1	1.6	48.3	3.1	1.8	41.9
Ash [a]	9.7	9.7	0	9.7	9.7	0	9.6	9.6	0

[a] Analyses carried out on separate samples.

Determination of the quantity of the individual constituents in each fraction was not made. In the larger fractions the variations in the losses the three cuttings were probably due to differences in the amounts of easily fermentable substances.

The types of micro-organisms in the fermenting hay greatly influenced the decompositions. The ground hay was very porous, hence it favored the rapid development of fungi, especially actinomycetes, which are capable of rapidly decomposing organic matter. Slimy masses of bacteria were also present in the fermenting hay although a more compact medium would have favored their development. The microbial decompositions were probably accelerated by the high nitrogen content in each cutting.

SUMMARY

A study was made of the influence of chemical composition on the microbial decomposition of successive cuttings of alfalfa hay. Data on the microbial decomposition of each cutting were obtained by measuring the daily evolution of carbon dioxide during a test period of 30 days, and by chemical analyses before and after fermentation.

Of the three cuttings of hay tested from the 1931 and 1932 crops, the third cutting underwent the greatest decomposition, the first cutting was next in order, and the second cutting showed the least decomposition.

The greatest variation in the rate of decomposition occurred during the first 10 to 12 days of the fermentations, after which it gradually decreased to the end of the experiment.

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PRESS FLUID FROM HEATED BEEF MUSCLE [1]

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INTRODUCTION

Juiciness, which is dependent upon the fluid content of muscle, is an important factor in palatability of meat, and quality is closely associated with palatability. Howe [2] describes juiciness in meat as its readily expressible liquid.

A grading chart with descriptive terms for scoring factors of palatability in meat has been developed by the cooking committee of the cooperative meat investigations committee.[3] This chart grades quantity of juice in meat as "very juicy", "moderately juicy", "slightly dry", "dry", "very dry", and "extremely dry." Quality of juice is graded as "very rich", "rich", "moderately rich", "slightly rich", "perceptible", "slightly perceptible." Grading the quantity and quality of juice in meat by such a method depends upon individual differences and standards of the judge. More accurate methods of determining both quantity and quality of juice are necessary for scientific experimentation in order that data of different investigators may be definitely compared.

The literature describes no definite method for pressing the fluid content from muscle, and little data can be found dealing with this pressed fluid.

This investigation was undertaken to develop a laboratory method for pressing out muscle fluid, by which different meat samples might be given a relative rating for quantity of press fluid or juice.

The purposes of this study were (1) to develop an apparatus for the removal of press fluid from roasted beef muscle; (2) to standardize methods for determining the percentage of press fluid and the ratio between press fluid and dry matter, and for obtaining press fluid for chemical analysis; (3) to use the standardized methods for studying press fluid in two beef muscles, psoas major, and biceps femoris; and (4) to compare the moisture, ether extract, and nitrogen contents of the press fluid from roasted beef muscle under pressure for two periods of 5 minutes and 20 minutes.

APPARATUS AND MATERIALS

The apparatus used in this study is called a "pressometer" (fig. 1). The term "press fluid" is used in preference to "juice" since in meat studies juiciness is graded by the individual's reaction when meat is eaten and probably includes not only fluids which are present in meat, but depends also upon the flow of saliva stimu-

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lated by meat extractives. Therefore, "press fluid" is used to designate the fluid consisting of moisture plus the soluble material plus the colloidal fraction that is pressed from muscle by the pressometer.

The pressometer (fig. 1) consists of a heavy cast-iron base with an attached motor. The muscle sample is wrapped in filter cloth (fig. 3) and placed in a brass tray (fig. 2) which is fitted into a grooved platform in the apparatus. Starting the motor, the platform with
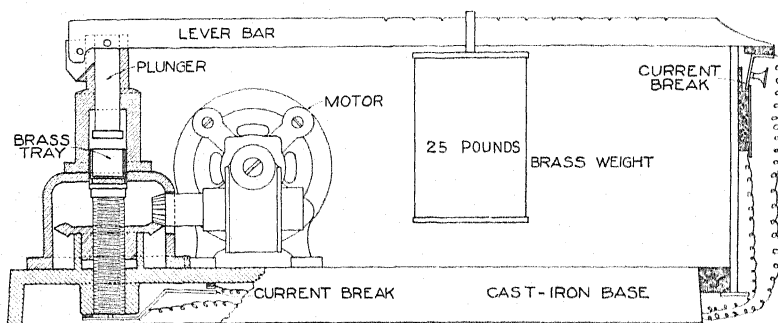
FIGURE 1.—Cross section of the pressometer, giving detail of working mechanism.

tray is driven upward by means of a large screw until it reaches a stationary plunger, the bottom of its base having the same dimensions as the inside of the tray. A 25-pound brass weight, which is suspended from the lever bar, exerts pressure on the sample. The pressure may be varied from 250 to 500 pounds by placing the weight at different points on the lever. When the desired pressure has been attained, the lever arm is lifted, breaking the current, and automatically arresting an increase in pressure. The actual time of pressure is checked by an automatic timer or stop watch. To carry the platform downward, the switch is reversed.

Samples were taken from beef roasts averaging 1½ pounds, from the left and right sides of the same animal. The roasts were stored in an electric refrigerator at 4° to 6° C. until used for cooking.

The rump was removed

FIGURE 2.—Brass tray for holding muscle sample.

from the wholesale round by cutting parallel and ventral to the pelvic bone (aitch bone). On the medial side (inside or top round) at this point, there are two large muscles, the posterior one being the semimembranosus muscle, immediately in front of which lies the adductor muscle from which samples were taken for the removal of press fluid.

Roasts were prepared according to the method given in the quality tests officially accepted by the cooking committee of the cooperative meat investigation committee,[4] but were not seared. The exterior

[4] ALEXANDER, L. M., CLARK, N. G., and HOWE, P. E. See footnote 3.

fat was removed, and the meat wiped with a damp cloth; it was then weighed, and all data for computing losses were recorded. The roast was tied with a string so that it was cylindrical in shape. The center of the bulb of a weighed, straight thermometer was inserted in the center of the roast. The meat was placed on a heavy wire rack, the rack being 1.25 cm from the bottom of the pan, in a weighed sheet-iron roasting pan (23.8 by 18.4 by 6 cm). The pan was set crosswise in the oven which was preheated to 125° C. The meat was roasted until the thermometer registered 60°.

STANDARDIZATION OF METHOD FOR USING PRESSOMETER

The first work on pressing muscle was done by placing the meat in a tray (2.5 by 2.7 by 0.8 cm) with a sieve bottom, the press fluid being collected in a tray placed directly under it. Because of the small size of the sample with which it was necessary to work, much of the fluid was lost either by evaporation or by adherence to to the tray, and small quantities of the solid part of the muscle were pressed through the sieve. Later, a tray with a slight metal elevation (height 0.3 cm) in the center was used, thus forming a groove around the edge, where the fluid could collect. This method, also, proved to be inadequate since it was difficult to press all of the fluid from the edges of the muscle and to remove it from the tray; therefore, it was decided to wrap the sample in a piece of cloth.

After trying different kinds, sizes, and shapes of cloth for wrapping the muscle sample, it was decided that unsized filter cloth, cut cross shape (fig. 3), gave the best results. The crosses were cut from the filter cloth after it had been boiled 10 minutes in distilled water and then dried.

FIGURE 3.—Cloth cross for wrapping muscle sample.

For sampling, a slice of meat was cut from the center of the roast with a sharp thin-bladed knife. A mechanical gage was used for determining the exact thickness. In order to obtain the most desirable thickness for cutting the meat, slices of different thicknesses (2.5, 1.87, and 1.25 cm) were tried. Those cut 2.5 cm thick were too large and showed that all the press fluid was not absorbed by the cloth. The press fluid from the 1.87-cm and 1.25-cm slices was absorbed by the cloth. It was decided to use the 1.87-cm slice in order to obtain more easily a sufficient quantity of fluid for chemical analysis.

A brief study was made of metal borers of different sizes and shapes. Two round borers (one, 1.27 cm in diameter, and the other, 2.4 cm in diameter) and a square borer (1 cm square) were used. The round borers were more easily used than the square one. The round borer 1.27 cm (fig. 4) in diameter was chosen, as the small sample had a more uniform structure of muscle fibers than the larger one.

Experimental work was carried out using different pressures for 10 minutes. The average [5] of press fluid pressed out by 112 pounds pressure was 43.90 percent; by 168 pounds pressure, 49.73; by 226.5 pounds pressure, 50.26; by 250 pounds pressure, 56.84; and by 500 pounds pressure, 58.61 percent. The data obtained showed that increased pressure yields more press fluid. Two hundred and fifty pounds pressure was selected because it was found that this pressure could be used also with chicken muscle, which is short fibered. Some of the solids of this tender muscle were pressed into the cloth when 500 pounds pressure was used.

To determine the optimum length of time for the removal of press fluid, four periods were used: 5, 10, 15, and 20 minutes. The mean press fluid for seven samples was as follows: 5 minutes, 49.00 percent; 10 minutes, 49.28 percent; 15 minutes, 50.64 percent; and 20 minutes, 50.66 percent.

Since there was not a great variation in the mean percentage of press fluid obtained from these different periods, the 10-minute period was used, as it allowed sufficient time for weighing samples and less time for evaporation.

The average press fluid in nine muscle samples was 49.93 percent, based on the difference between the weight of the original sample and the pressed muscle. The average press fluid in the cloth from the same samples was 48.98 percent, based on the weight of the original dry cloth and the cloth moistened with the pressed fluid. The slightly lower percentage of fluid in the cloth than in the muscle was probably due to a faster evaporation in the cloth.

To compare the quantity of press fluid from roasts of different temperature, samples were cut (1) at the time the roasts were taken from the oven, (2) 2 hours after taking from the oven, (3) 3½ hours after taking from the oven. The mean press fluid from roasts at approximately 55.5° C. was 49.64 percent; at 23.3°, 49.74 percent; and at 21.1°, 49.52 percent. From the mean percentages of press fluid obtained from samples of roasts at these different temperatures, it can be seen that there was not an appreciable difference in the total quantity. It seemed evident that all of the small samples of muscle were about the same temperature at the actual time of pressing. To prove this, thermocouples were used to obtain the temperature of the small samples from the roasts of varying temperatures, and it was found that by the time the sample was cut, weighed, and wrapped in cloth, the muscle had almost reached room temperature (21°). Since there was so little difference in the quantity of press fluid, all roasts were cooled to 40° before sampling.

FIGURE 4.—Borer, 7.6 cm in length and 1.27 cm in diameter, used for cutting muscle sample.

METHODS SELECTED FOR OBTAINING PRESS FLUID FROM ROASTED BEEF MUSCLE

From the preliminary work the following were chosen as desirable methods for obtaining press fluid from roasted beef muscle and were used in succeeding experiments.

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[5] The percentages press fluid for 112, 168, and 227.5 pounds pressure were obtained by using an unimproved pressometer.

A slice 1.87 cm thick was cut from the center of the roast by means of a sharp thin-bladed knife, using a mechanical gage for determining the thickness of the slice.   Three adjacent samples were cut from the center of the slice with a round borer 1.27 cm in diameter (fig. 4). Each sample was transferred to a numbered, previously weighed, aluminum dish containing a piece of dry, weighed, unsized, shrunken filter cloth that was cut cross shape.   The dish with cloth and muscle sample was then weighed.   The sample was carefully wrapped and placed in the tray (fig. 2), which was inserted in the pressometer and allowed to remain for 10 minutes at a pressure of 250 pounds.   The muscle sample and the cloth were removed from the tray and placed in separate, dried, weighed aluminum dishes and weighed; samples were kept in the desiccator until ready for weighing.   Rapid work was necessary from the time the sample was cut to the last weighing, so as to avoid evaporation losses.   Forceps were used for all handling.

The percentage of press fluid in the muscle was found by dividing the weight of the press fluid by the weight of the muscle sample before pressing.   The weight of the press fluid was found by subtracting the weight of the pressed muscle sample from the weight of the unpressed sample.

METHOD USED IN OBTAINING RATIO OF PRESS FLUID TO DRY MATTER FROM HEATED MUSCLE

The method for obtaining the quantity of press fluid from heated muscle, as previously explained, was followed.   The two aluminum dishes with weighed samples of pressed muscle in one and the cloth containing the press fluid in the other were then dried in a Freas vacuum oven at 65° C.   The ratio of press fluid to dry matter was calculated by dividing the weight of the press fluid by the weight of the dry matter.   The dry matter includes both that in the residual muscle and that adhering to the cloth.

METHOD USED FOR OBTAINING PRESS FLUID FOR CHEMICAL ANALYSIS

For chemical analysis of the press fluid, samples of muscle were cut from the center slices of the roast.

The sample was set in the center of a weighed, cross-shaped filter cloth, wrapped, and placed in the pressometer and allowed to remain for 5 minutes at a pressure of 250 pounds; the pressed muscle was then discarded and the process was repeated, using the same piece of cloth with two other muscle samples.   The cloth was removed to a weighed corked bottle and placed in the desiccator.   When four pieces of cloth were similarly filled with press fluid, the bottle and cloth were weighed and the amount of press fluid was calculated.   This quantity of press fluid (5½ g or more) was sufficient to determine the ether extract, nitrogen, and moisture content.   The corked sample bottle, containing the filter cloth with pressed fluid, was kept in a cold room (− 14° to − 15° C.) until needed for analysis.

The sample, after being prepared for analysis, was cut in pieces approximately one-fourth inch square and was divided into five aliquot portions, each being placed in a weighed aluminum dish. The covered dishes were allowed to stand at room temperature for

15 minutes, then weighed. Moisture, ether extract, and nitrogen were determined by the official methods of analysis of the Association of Official Agricultural Chemists.[5]

EXPERIMENTAL DATA FROM USE OF THE PRESSOMETER

The three methods of experimentation were used for comparing (1) the press fluid in two beef muscles, one tender (psoas major) and one less tender (biceps femoris), and (2) the moisture, ether extract, and nitrogen in the press fluid from roasted adductor muscle when the samples were kept under pressure for two periods, 5 minutes and 20 minutes.

RATIO OF PRESS FLUID TO DRY MATTER IN ROASTED BEEF

Table 1 presents the mean grams of fluid per gram of dry matter from a tender muscle (the psoas major) of roast beef, and a less tender muscle (the biceps femoris). The mean grams of fluid per gram of dry matter (1.847 for biceps femoris and 1.823 for psoas major) did not vary significantly, as determined by means of the t test [6]; t is 0.226 and P is between 0.90 and 0.80 which means that in 80 to 90 cases out of 100 this difference is due to chance.

TABLE 1.—*Mean [a] grams of press fluid per gram of dry matter in the muscles, biceps femoris and psoas major, from roasted beef*

Roast no.	Biceps femoris muscle	Roast no.	Psoas major muscle
1	1.620	9	1.815
2	2.076	10	1.660
3	1.583	11	1.336
4	1.946	12	2.160
5	1.903	13	2.013
6	1.796	14	1.596
7	2.100	15	1.926
8	1.753	16	2.080
Mean	1.847	Mean	1.823

[a] From 3 samples from center slice of roast.

MOISTURE, ETHER EXTRACT, AND NITROGEN IN PRESS FLUID FROM ROASTED BEEF WHEN SAMPLES WERE UNDER PRESSURE FOR PERIODS OF 5 AND 20 MINUTES

The percentage of moisture in press fluid from the roasted adductor muscle of beef under pressure for periods of 5 and 20 minutes is given in table 2.

The mean percentage of moisture in the press fluid when the samples remained under pressure for 5 minutes was 89.974, and for 20 minutes 88.757, showing slightly less moisture after it had been under pressure for 20 than for 5 minutes. This slight difference may be due to greater evaporation when the sample remained under pressure for the longer time; t is 1.640 and P is between 0.20 and 0.10, which means that in 10 to 20 cases out of 100 this difference is due to chance. These data indicate that duration of pressing is not likely to affect the percentage of moisture in press fluid.

[5] ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. Compiled by the committee on editing methods of analysis ... Ed. 3, 593 pp., illus. Washington, D.C., 1930.

[6] FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed. 4, rev. and enl., 307 pp., illus. Edinburgh and London. 1932.

TABLE 2.—*Percentage of moisture, ether extract, and nitrogen in press fluid from roasted adductor beef muscle under pressure for 5-minute and for 20-minute periods*

MOISTURE

5 minutes		20 minutes	
Roast no.	Moisture	Roast no.	Moisture
18	90.96	19	88.46
20	89.37	21	89.52
22	90.32	23	89.05
24	90.44	25	90.67
26	90.39	27	90.72
28	89.28	29	90.54
30	89.53	31	87.94
32	91.15	33	90.38
34	90.49	35	88.52
36	86.80	37	90.01
38	99.71	39	91.08
40	89.24	41	86.49
42	82.25	43	78.83
44	89.71	45	90.38
Mean	89.974	Mean	88.757

ETHER EXTRACT

5 minutes		20 minutes	
18	1.81	19	3.14
20	2.39	21	2.82
22	2.53	23	2.86
24	2.00	25	2.48
26	1.05	27	2.16
28	3.65	29	3.07
30	2.87	31	1.87
32	1.61	33	.97
34	1.70	35	1.86
36	3.24	37	2.61
38	1.74	39	.73
40	2.82	41	7.18
42	7.32	43	7.07
44	1.26	45	1.87
Mean	2.571	Mean	2.906

NITROGEN

5 minutes		20 minutes	
18	1.040	19	1.020
20	.980	21	.950
22	.984	23	.965
24	1.021	25	.946
26	.950	27	.927
28	.938	29	.942
30	.997	31	.985
32	.975	33	.906
34	1.005	35	.925
36	1.002	37	.950
38	.888	39	.866
40	1.000	41	.957
42	.963	43	.964
44	1.010	45	.954
Mean	.982	Mean	.947

The quantity of fat in press fluid may be an indication of the quality of press fluid or juice in meat. In order to determine whether time of pressing had any effect on the quantity of fat, the ether extract obtained from press fluid of roasted adductor beef muscle under pressure for 5 minutes was compared with that obtained under 20 minutes' pressure (table 2). In the case of ether extract, the 5-minute period gave a mean percentage of 2.571, and the 20-minute period one of 2.906, t being 0.914 and P between 0.40 and 0.30,

indicating that the difference is not significant.[7] A longer time under pressure is not likely to influence the final percentage of ether extract obtained from the press fluid, since muscle fat solidifies when cool.

The mean percentage of nitrogen (table 2) in the press fluid from the roasted muscle was found to be significantly higher after a 5-minute expression period than after a 20-minute period, being 0.982 percent for the former and 0.947 percent for the latter. The t test applied to this difference gave $t = 4.843$, and P is less than 0.01. One might assume that more nitrogen would be obtained in press fluid when the longer period is used, because of the possible pressing out of colloidal material into the cloth. A possible explanation of the fact that the greater percentage of nitrogen appears during the 5-minute period than during the 20-minute period, is that the soluble nitrogen is pressed out at the beginning of the period. Gortner, Lawrence, and Harris[8] in their work on plant tissue, state that in some instances the fluid extracted by continuous pressing, without rearrangement of the tissue mass, may become less and less concentrated. This was found to be true in a series of extractions from cabbage leaves.

SUMMARY

An apparatus called the "pressometer", developed to press fluid from heated beef muscle, is described.

Methods are explained for using the pressometer, for determining the percentage of press fluid and the ratio of press fluid to dry matter in heated beef muscle, and for obtaining press fluid from heated beef muscle for chemical analysis.

From this study the following observations may be made on the basis of statistical analysis:

The mean percentages of ether extract and moisture in the press fluid from the adductor muscle from roasted beef, did not vary significantly when samples were under pressure for 5 and for 20-minute periods.

The mean percentages of nitrogen in the press fluid from adductor muscle from roasted beef varied significantly when samples were under pressure for 5 and for 20-minute periods, the greater percentage of nitrogen appearing during the 5-minute period.

The mean percentages of press fluid from the muscles psoas major and biceps femoris from roasted beef did not vary significantly when the grams of press fluid per dram of dry matter were compared.

[7] FISHER, R. A. See footnote 6.
[8] GORTNER, R. A., LAWRENCE J. C., and HARRIS, J. A. THE EXTRACTION OF SAP FROM PLANT TISSUES BY PRESSURE. Biochem. Bull. 5: 139–142. 1916.

CARBON DIOXIDE FORMATION BY CLEAN AND SCABBY POTATOES [1]

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INTRODUCTION

Losses in weight of potato tubers in storage are due to both transpiration and respiration, but much the greater losses are due to the former. The checking of such losses by the tubers is largely through the skin. The skins of scabby potatoes are altered considerably by a parasitic growth which stimulates the formation of a loose and very permeable covering. Such tubers lose weight reapidly after removal from the soil. The gradual healing of the abnormal skin areas after the tubers have been in storage for some time tends to check these heavy initial losses and to restore the tubers almost to the same condition as healthy ones.

PREVIOUS WORK

The relation of respiration to loss of weight in potatoes, apples, carrots, and other fruits and vegetables has been discussed in considerable detail by various authors. Especially important in this connection is the work of Appleman, Kimbrough, and Smith (1)[3] on the physiological shrinkage of potatoes in storage, and that of Kimbrough (5) on the respiration of potatoes during storage and transportation. Some interesting work on the internal gases of potato tubers has been reported by Magness (7). A detailed account of the relative losses in weight of clean and scabby tubers is given in an earlier report by the present writer (6).

The method used by all these investigators except the last mentioned is to pass carbon-dioxide-free air through a vessel containing a weighed lot of potatoes. This air sweeps out the carbon dioxide formed by the tubers. The air with its accumulated carbon dioxide is then passed through a solution such as barium hydroxide. A titration of this solution at the conclusion of the run gives the amount of carbon dioxide evolved.

The method used by the author (6) was the sampling method, the determination of the percentage of carbon dioxide in the 10-cc sample being determined by the Haldane apparatus. By this method the respiration of 10 pounds of potatoes kept under bell jars between October 4 and March 29 was only 2.471 for clean tubers or 3.757 for scabby ones. The temperature during much of this storage period was only 2° C. Although at the beginning of the season it has been 12°, it dropped rapidly to about 3° to 4°. The total respiration loss of the tubers kept by Kimbrough (5) is not given, but from his graph the

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loss between October 1 and February 7 (129 days) for a kilogram of
tubers kept at 22° C. (71.6° F.) appears to be about 17. The respira-
tion at 40° of Green Mountain tubers, according to Wright (9), was
in milligrams per kilogram-hour on November 7, 5.11; on January 17,
3.57; and on March 24, 2.93. At these rates the total loss per kilo-
gram would be for the 5 storage months, November to March,
inclusive, about 15 g.

In a recent publication Smith (8) reviewed much of the literature
and added extensive data on the effect of temperature, humidity,
injury, depth of layer, etc., on potatoes in storage. Ordinary storage
cellars were used in some of the experiments, and the loss in weight
from respiration during a 7-month period ranged from 0.40 to 0.67
percent of the total weight of the tubers. The loss from respiration
of mechanically injured tubers, while large at first, dropped rapidly
until it was no greater than that of uninjured ones. The depth of the
potatoes in the bin had little effect on the carbon dioxide formation,
although it was somewhat higher in the bottom layers than in the top
ones. The difference was noticeable only at the end of the storage
season when sprouting was probably starting. Smith does not discuss
the relative merits of the methods used for respiration determination.

PURPOSE OF THE WORK

The purpose of the experiments here reported was primarily to
ascertain the loss by respiration of scabby potatoes as compared
with that of clean ones after various periods in storage, and to compare
the results obtained by the aspirator method with those obtained by
the sampling one.

METHODS

Four lots of tubers as nearly alike as possible in size and number
were placed under bell jars. Each lot weighed approximately
1,000 g. Two of the lots were composed of clean tubers and two of
scabby tubers. The jars contained 8 liters of air after the tubers
had been placed in them. To prevent the growth of mold, the tubers
were immersed for 1 hour in a weak formaldehyde solution before they
were placed in the jars, and fresh tubers were substituted if decay
appeared. The jars were kept in a basement room the temperature
of which ranged from 13° to 15° C.

The amount of carbon dioxide evolved was measured by (1) the
aspirator method, and (2) the sampling or Haldane method. The
amount of carbon dioxide formed in a jar of clean and in one of scabby
tubers was determined by each method.

When the aspirator method was used laboratory air, freed from
carbon dioxide and moisture (fig. 1), was passed through the bell jar
containing the tubers and then into a small Erlenmeyer flask contain-
ing 100 cc of 0.1650 normal barium hydroxide solution. After the
air in the bell jar had been passed for 24 hours through this solution,
it was titrated with 2/10 N oxalic acid and the amount of carbon
dioxide computed.

An analysis of the air inside bell jars containing clean or scabby
tubers was made daily by the use of a Haldane apparatus during the
early part of the storage season and usually at intervals of 2 days
during the late fall. The tubers were left under the jars for periods of

5 to 7 days during the winter, the carbon dioxide being allowed to accumulate in the jar. The air in the bell jars was thoroughly mixed each time by the aid of a celluloid fan operated from outside to insure a homogeneous mixture.

The intercellular gas of the tubers used in the experiments was removed from a number of the lots. The method of extraction was
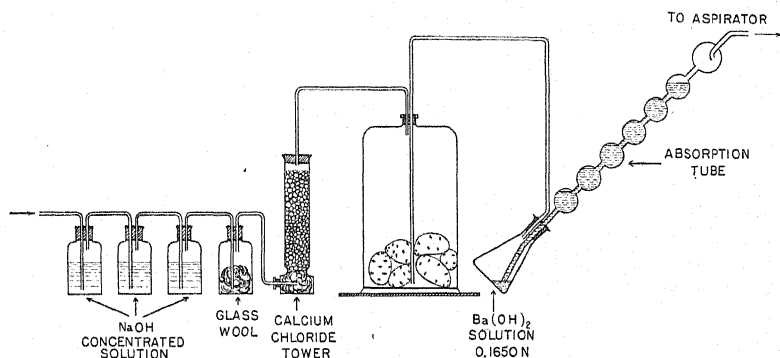
FIGURE 1.—Diagram of apparatus used for obtaining the carbon dioxide production by the use of a continuous current of carbon-dioxide-free air.

that described by Magness (7), and the determination of carbon dioxide and oxygen was made by a modified Henderson apparatus (fig. 2). The modification consisted in the use of a 3-cc calibrated tube instead of the usual 10-cc tube. Three cubic centimeters of gas was usually all that could readily be obtained. Eight plugs, 1 or 2 from each tuber, were placed in the mercury. The modified Henderson appa-
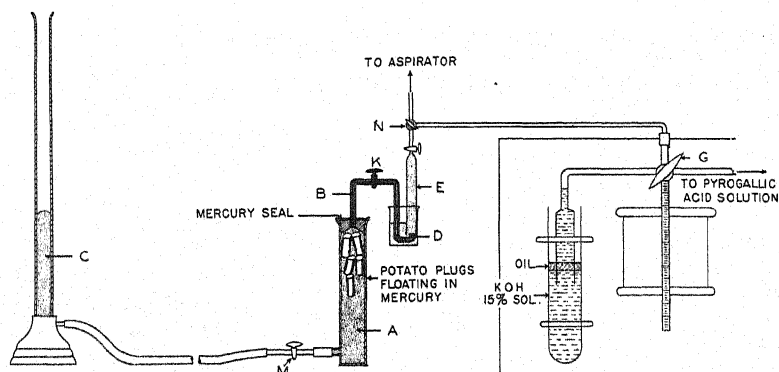
FIGURE 2.—Diagram of apparatus used for the extraction of internal gases from the potato tuber and for the determination of the percentage of carbon dioxide and oxygen in the 3-cc tube employed in the modified Henderson apparatus. *A*, Jar filled with mercury and potato plugs; *B*, tube filled with mercury; *C*, jar filled with mercury, connected by heavy rubber tubing with jar *A*, controlled by stop cock *M*, jar *C* can be raised and lowered; *D*, beaker filled with mercury in which is placed the gas-collecting tube *E*, controlled by stopcock *K*; *N*, stopcock from collection tube *E*, to aspirator or to CO₂ and O₂ apparatus; *G*, three-way stopcock to calibrated CO₂ apparatus immediately below or to pyrogallic acid solution on right but not shown in the figure.

ratus is not so accurate as the Haldane, but it is probably not more than 1 percent in error.

To determine the amount of carbon dioxide that would accumulate in air-tight potato bins, two large cylindrical, galvanized iron tanks, each 5½ feet high, were filled with clean or scabby potatoes. Before the potatoes were placed in the bins, glass tubes with a bore of about

2 mm (barometer) were placed in each so as to tap the air between the tubers at the 5-, 4-, 3-, 2-, and 1-foot levels. The upper end of each tube was closed by a pinchcock. Samples of the storage air at the various levels could be drawn through the tubes without disturbing the other air or the tubers.

DATA

LOSS OF CARBON DIOXIDE FROM CLEAN AND SCABBY TUBERS

Four lots of tubers were carried through the storage season under bell jars. These tubers were changed every 2 to 3 weeks from a supply kept under the same conditions. Two jars contained clean tubers and two contained scabby ones. The respiration in a clean and in a scabby jar was measured by the absorption-aspirator method and similar lots were measured by the sampling method. The experiments were continued from October 11, 1930, to March 19, 1931, a total storage period of 159 days. A summary of the results, presented in tables 1 and 2, shows that the general effect of scabbing was to increase the loss of carbon dioxide except during the last month or so of storage. The loss as determined by the absorption method was almost twice as great as that by the sampling method.

TABLE 1.—*Carbon dioxide production (milligrams) by clean and scabby potatoes during the storage season, as determined by the absorption and sampling methods*

Month	Average production of CO_2 per kilogram-hour				Average production of CO_2 per kilogram per day			
	Absorption method		Sampling method		Absorption method		Sampling method	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
October	8.8	10.0	6.7	8.7	211.2	240.0	160.8	208.8
November	4.1	6.0	2.7	4.0	98.4	144.0	64.8	96.0
December	3.3	5.9	1.6	2.2	79.2	141.6	38.4	52.8
January	4.2	4.9	2.1	2.4	100.8	117.6	50.4	57.6
February	5.5	5.1	2.7	2.8	132.0	122.4	64.8	67.2
March	9.3	6.7	4.0	3.5	223.2	160.8	96.0	84.0

TABLE 2.—*Total carbon dioxide production (grams per kilogram) by clean and scabby potatoes, by the month or part of the month, and also for the entire storage season, as determined by the absorption and sampling methods*

Month	Absorption method		Sampling method	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
October (20 days)	4.224	4.800	3.216	4.176
November	2.952	4.320	1.944	2.880
December	2.455	4.390	1.190	1.637
January	3.125	3.645	1.562	1.785
February	3.696	3.427	1.814	1.881
March (19 days)	4.241	3.055	1.824	1.596
Total (159 days)	20.693	23.637	11.550	13.955

The results are a reflection of the pathology of the scabby tubers and are an interesting addition to the physiology of abnormal growths. The loss of carbon dioxide was relatively heavy from the scabby

tubers during the early part of the season (October to February), but by February the scab lesions had healed so thoroughly that the skins of the scabby tubers were as impermeable as those of the clean ones. During March the scabby tubers should apparently have had an even better protection against the loss of carbon dioxide than the clean ones. The fact that the clean tubers produced more carbon dioxide late in the season was due to their earlier germination, as table 3 shows.

TABLE 3.—*Carbon dioxide production (milligrams per kilogram-hour) by clean and scabby potatoes during 3 periods in March, as determined by the absorption and sampling methods*

Period	Absorption method		Sampling method	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
Mar. 1 to Mar. 5	5.8	6.0	2.8	2.8
Mar. 9 to Mar. 13 (sprouting in clean tubers begun)	10.2	6.3	3.3	2.7
Mar. 15 to Mar. 19 (sprouting in clean and some in scabby tubers advanced)	11.7	7.8	6.4	5.6

Scab spots delay germination from 1 to 2 weeks, and during that time the carbon dioxide production is greater from clean tubers with their large sprouts.

COMPARISON OF RESULTS OBTAINED BY THE TWO METHODS

As compared with the sampling method the absorption method gave a uniformly higher production of carbon dioxide from both clean and scabby tubers (table 4). The conditions under which the comparisons were made should be noted. When the absorption method is used all the air from the bell jar is swept out by the air current passing over the tubers; when the sampling method is used the air over the tubers remains the same and the carbon dioxide produced remains in it. However, the amount of carbon dioxide in the jars never approached 2 percent, the limit of determination by the Haldane apparatus.

TABLE 4.—*Daily carbon dioxide production (milligrams per kilogram-hour) by clean and scabby potatoes for the days after the tubers had been aired and then replaced under the bell jars, or other tubers substituted, as determined by the absorption and sampling methods*

Date	Absorption method		Sampling method	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
Dec. 9	2.9	6.1	2.0	4.2
10	3.6	6.0	1.5	1.9
11	5.1	6.1	1.5	2.2
12	3.4	6.0	1.4	2.0
13	3.3	5.9	1.3	1.8
Jan. 7	3.7	5.3	3.1	3.5
8			1.8	2.0
9	3.9	5.0	1.8	1.8
			1.7	1.7
29	4.6	4.3	3.3	3.6
30			2.3	2.5
31	4.5	4.1	2.0	2.3
Feb. 1			1.8	2.2

The amount obtained with the Haldane apparatus the first day after the tubers were placed under the bell jars was similar to that obtained by the other method, but the drop was abrupt after that time and the amount remained relatively small. The tubers used were stored in the same room as the bell jars and had been kept there for at least 1 week before they were used.

Every period after the changing of the tubers showed a similar slump in the rate of respiration as indicated by the Haldane apparatus. The carbon dioxide in the other bell jars must have had the tubers as its source. The internal, intercellular gases percolate out through the lenticels from the inside of the tubers. With this point in mind, a measurement of the internal gases of the tubers under each of the jars was made at intervals after a series of readings had been taken for carbon dioxide formation (tables 5 and 6).

TABLE 5.—*Carbon dioxide production (milligrams per kilogram-hour) by clean and scabby potatoes on days upon which the internal gases were also analyzed, as determined by the absorption and sampling methods*

Date	Absorption method		Sampling method	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
Nov. 5	5.8	7.8	4.3	8.1
Nov. 27	2.3	4.1	1.3	1.5
Dec. 18	3.4	6.3	1.3	2.0
Jan. 17	4.0	5.1	2.1	2.2
Feb. 16	4.4	4.2	2.3	2.4
Mar. 4	6.1	6.6	1.2	1.0
Mar. 21	11.0	8.2	8.6	8.4

TABLE 6.—*Percentage of carbon dioxide and of oxygen in the internal gases of stored potato tubers after the completion of the respiration tests*

Date	After absorption method				After sampling method			
	CO_2		O_2		CO_2		O_2	
	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes	Clean potatoes	Scabby potatoes
Nov. 5	35.6	28.6	7.8	12.0	40.0	56.1	11.0	6.9
Nov. 27	35.5	33.1	8.6	9.0	42.6	52.8	8.0	5.7
Dec. 18	29.8	28.3	6.8	10.7	35.9	46.1	11.7	9.7
Jan. 17	32.5	36.5	8.1	6.9	35.3	43.7	9.5	8.7
Feb. 16	28.2	32.9	10.4	11.0	37.1	42.7	8.6	6.2
Mar. 4	22.7	55.3	7.0	10.1	33.8	54.8	5.2	9.9
Mar. 21	45.6	58.3	3.4	8.3	55.1	60.1	4.1	5.9
Average	32.84	39.00	7.44	9.71	39.97	50.90	8.30	7.57

When determinations were made by the sampling method the amount of carbon dioxide remaining in the internal gases of the tubers was higher in all except one instance (when it was about the same) than it was when the absorption method was used (table 6), indicating that in the passage of the air through the bell jar for 24 hours, a considerable percentage of the internal carbon dioxide found its way out of the tuber. Determinations by the absorption method

showed that during November and December, before the scab lesions had completely healed, the scabby tubers allowed more carbon dioxide to pass out than the clean ones, i.e., the percentage remaining within them was smaller. In January, however, and the months following, the percentage remaining inside the scabby tubers was higher than in the clean ones.

AMOUNT OF CARBON DIOXIDE REQUIRED TO CHECK RESPIRATION

The total accumulated carbon dioxide under the bell jars used for sampling never approached 2 percent. Trials made to determine the percentage necessary to check respiration, not to prevent it entirely, indicated that 7 to 9 percent was sufficient and that the acidity of the juice changed from about pH 6.2 to pH 5.8. Such a high percentage of carbon dioxide could probably never develop in a storage bin. The 7 percent carbon dioxide slowed down respiration to a minimum, but at least 16 percent of carbon dioxide was required to prevent it entirely. Kidd (4) found that 20 percent would not only stop respiration but would also prevent sprouting.

AMOUNT OF CARBON DIOXIDE ACCUMULATING AT BOTTOM OF BINS

In order to determine the quantity of carbon dioxide that would accumulate in a bin, potatoes were stored in two large open-topped cylinders, clean tubers in one and scabby tubers in the other. Before the potatoes were put into the cylinders, rods of barometer glass were so suspended that air could be withdrawn from depths of 1, 2, 3, 4, and 5 feet. When these experiments were started about March 15, 1931, the storage temperature was approximately 38° to 40° F. Samples were taken weekly, but the percentages of carbon dioxide remained the same as that of the surrounding air until April 15. By that time sprouting was common on both clean and scabby tubers, and the temperature rose to 50° to 52° and the percentage of carbon dioxide in the bottom of the cans rose to 0.7 percent. In 1933 the experiment was repeated, starting February 9 and continuing until May 3. The readings from clean and scabby potatoes were approximately the same, 0.4 percent at 1 foot, 1.3 percent at 2 feet, 1.8 percent at 3 feet, 1.7 percent at 4 feet, and 1.8 percent at 5 feet.

The percentages which appear in the later readings are higher than those of Smith (8), but Smith's data are from tubers stored in bins that were not necessarily airtight, whereas the data recorded in this work were from tubers stored in cans. The top layers even in a can of potatoes, however, interpose only a small obstacle to the outward diffusion of gases formed inside the can. If such a formation is slow, as occurs from potatoes stored at low temperatures, the diffusion may keep pace with the formation in a bin that is only 5 feet in depth. The holes between the tubers, as was found by actual measurement, represent 17.7 percent of the surface area of the large potatoes and 14.8 percent that of the small ones. According to Brown and Escombe (2), if 11.34 percent of a septum area is composed of holes, the diffusion is 60 percent that of an open vessel. The irregular shape of the holes undoubtedly added to their diffusion efficiency as compared with circular holes. The 15 to 18 percent area of the interstices between the tubers would accordingly mean a diffusion equal probably to at least 70 percent of that of an open vessel.

Other factors, such as shape of pores and depth to which they are embedded, velocity of wind stirring, and the percentage of any gas in the storage chamber, affect the rate of gas diffusion (*3*), but these factors were not investigated in the present work as the area of a tortuous passage from the bottom of a container filled with potatoes would be difficult to determine.

DISCUSSION

Respiration was always less under the bell jars tested by the Haldane apparatus than in those through which the carbon-dioxide-free air was passed. This may be explained by the diffusion of gases through the pores of an impervious membrane. The skin of the potato tuber is the membrane broken by the openings which are the lenticels. The chamber containing the CO_2 is the potato.

Brown and Escombe (*2*) have shown that around each pore an area of gas collects; very dense at the pore, gradually becoming more diluted as the distance from the pore increases. This is their so-called "shell" of gas. Exactly the same shell of CO_2 must occur over each pore, i.e., over each lenticel of the tuber. Let us see how these shells of gas behave under the two treatments.

In the aspirator method, when air, free of CO_2, passes over a lenticel, at the entrance to which the percentage of CO_2 is approximately, let us say 28.6 percent, the shell of carbon dioxide is swept away, leaving a very sharp gradient between the internal gas of the tuber and the air rushing by the opening. The CO_2 is densest at the center of the tuber away from these pores. The CO_2 rushes out of the tuber, is swept along and measured as part of the respiration. This lowers the amount of CO_2 remaining inside the tuber, as shown in table 6. The apparent respiration CO_2 by this method therefore includes (1) the carbon dioxide in the jar; (2) the carbon dioxide surrounding the pores, i.e., the shells; and (3) part of the carbon dioxide which is normally within the tuber but which rushes out through the pores when air is swept over them for 24 hours.

In the sampling method, the air inside the bell jars is still. Obviously a larger shell of diluted CO_2 can be formed over the pore. The preparations are made for taking the sample. The air is stirred by a celluloid fan, breaking up the shells around the pores and distributing the CO_2 throughout the air of the bell jar so as to make a homogeneous gaseous mixture. The 10-cc sample is then taken immediately, the whole process involving only a few minutes. Some of the internal CO_2 rushes out through the pore, but the amount is negligible because the operation is of such short duration. The apparent respiration by the sampling method, therefore, includes CO^2 from only two sources: (1) that which is in the bell jar entirely free from the tubers; and (2) the shells of CO_2 distributed throughout the jar by the celluloid fan.

A comparison of tables 5 and 6 will show the correctness of the data on which the foregoing explanation is based. A discussion of the physical phase may be found in Brown and Escombe's paper (*2*) and the effect of currents of air in Huber's (*3*).

The aspirator method would most properly be used for studies on apple storage; the sampling method would be better for potatoes and root crops. Neither of these methods would give absolute correct-

ness. The same conclusion on potato storage may be drawn from this work that Smith (8) drew from his, namely, that carbon dioxide will never collect in sufficient quantity to make ventilation in a storage cellar necessary.

SUMMARY

The respiration records of four lots of potatoes were studied through a storage period of 159 days. Two of these lots were composed of clean tubers and two of scabby tubers.

The respiration rate was obtained (1) on one lot each of clean and scabby tubers by passing carbon-dioxide-free air through the bell jars in which they were kept, the carbon dioxide being absorbed by an alkaline solution; and (2) by withdrawing 10-cc samples from one lot each of the clean and scabby tubers and determining the percentage of carbon dioxide by the use of a Haldane apparatus.

During the first month of the storage period the respiration rates of the scabby tubers were much higher than those of the clean tubers as the cork layers under the scab lesions were not so impervious to gases as the skin of the uninjured ones. The rates tended to become equalized during January and February, but after sprouting began in March the clean tubers respired more than the scabby ones, as sprouting was delayed for some time (7 to 10 days) by the scab lesions.

The respiration rates obtained by passing carbon-dioxide-free air through bell jars containing tubers were uniformly higher than those obtained by taking samples of the air in bell jars containing tubers. The reason suggested for the higher respiration rates obtained by the absorption method is that the flow of air through the jar removes the carbon dioxide not only from around the tubers but also from the gases held in the intercellular spaces. It is also suggested that the checking of respiration from tubers under the bell jars used in the Haldane apparatus tests may have been due to "shells" of carbon-dioxide-laden air around the opening of the lenticels. These shells are not disturbed in the air of the bell jar and check the outward diffusion of carbon dioxide.

The small amount of carbon dioxide which had accumulated inside the bell jars used for the Haldane apparatus tests was not sufficient to check respiration, 7 to 9 percent being required to check it.

The amount of carbon dioxide which appeared at the bottom of a special storage bin in which the tubers were 5 feet in depth was practically that of the surrounding air during the storage season and never rose until the tubers began to sprout, when as high as 1.8 percent occurred at the bottom with a temperature of about 50° F. This diffusion of carbon dioxide from the surface of potato bins follows the laws of the diffusion of gases through pores. With large tubers 17.7 percent of the surface was pore area, and this surface offered little resistance to the diffusion of the carbon dioxide formed at the bottom.

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